UNIVERSITY OF ALBERTA

THE ISOLATION, CHARACTERIZATION AND METAL ION BINDING PROPERTIES OF THE α-SUBUNIT OF BOVINE BRAIN S-100a PROTEIN

BY

IRIS K.M. LEUNG

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA
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To my family

ABSTRACT

The S-100 proteins, S-100a$_0$ (αα), S-100a (αβ) and S-100b (ββ), belong to the family of EF hand calcium binding proteins. Previous studies indicated that the two principal isoforms, S-100a and S-100b, differ in their metal ion binding properties. In order to facilitate a comparison of the physico-chemical properties between the two highly homologous subunits, a preparative procedure was developed to isolate and purify the α-subunit of S-100a protein from its parent molecule, using anion exchange chromatography under denaturing conditions.

Both hydrodynamic and spectroscopic approaches were employed to elucidate the physico-chemical properties of the α-subunit. Sedimentation equilibrium measurements indicated the purified α-subunit to exist as a non-covalently linked dimer with a $\overline{M}_w$ of 21,370 ± 1000. Circular dichroism (CD), fluorescence and absorption difference spectroscopy revealed that the α-subunit undergoes a conformational change upon binding Ca^{2+} and Zn^{2+}, and the 2 metal ion binding sites appear to be different. Fluorescence measurements suggested that 2 Ca^{2+} binding sites exist on the α-subunit at pH 8.4, with apparent K_d values of ~10^{-4} M and 10^{-5} M, compared to a single low affinity site (10^{-4} M) at pH 7.5. The monovalent cation, K^+, was observed to modulate Ca^{2+} binding to the protein.

The single cysteine in the α-subunit was labelled with the fluorescent probe, Acrylodan. The results obtained from this study, as well as those from other spectroscopic techniques, indicated that the conformational change induced by Ca^{2+} binding to the protein was localized in the C-terminal region where Cys 85, Trp 90 and a hydrophobic domain were located. Zn^{2+}, on the other hand, did not perturb this region of the molecule to any great extent. Chemical modification of the single tryptophan with N-bromosuccinimide and possibly one of the 2 tyrosines at positions 26 and 74 with tetranitromethane, implied that

the native conformation of the protein was extremely sensitive to perturbation of these residues.

An earlier study indicated S-100α and S-100β bound and stimulated the *in vitro* activity of brain aldolase in a Ca^{2+} dependent manner. Prior to investigating the molecular basis of this interaction, enzymatic studies repeated in our laboratory indicated a small Ca^{2+} dependent stimulation of aldolase C activity only at abnormally high molar ratios of S-100 : aldolase (213:1). These findings, coupled with our inability to demonstrate a physical interaction between the two proteins using CD and affinity chromatography, suggest that such an interaction, if it occurs, is unphysiological.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr. C. M. Kay, for his patient guidance and encouragement throughout the course of my studies as well as a careful review of this thesis. In particular, I wish to thank Dr. R.S. Mani who has contributed invaluable scientific input to the project, as well as giving advice during the preparation of this thesis.

All the members in Dr Kay's laboratory have contributed to various aspects of this project and I am grateful for their assistance: to Dr. W.D. McCubbin for sharing his expertise in protein chemistry and reading the thesis, to Kim Oikawa for performing the circular dichroic measurements, to Les Hicks who operated the ultracentrifuge and expert help with the graphics and photography. The technical assistance of Toni Keri is also acknowledged.

Special thanks are due to Dr. L.B. Smillie for extending his use of his laboratory facilities for amino acid analysis, and to Mike Nattriss for his technical assistance in carrying out the analyzer runs.

I would like to thank Dawn Oare for helping with the typing of the thesis and to Vicki Luxton for explaining to me the intricacies of the "Microsoft Word" software.

The friendships that developed during my years in the department have a special meaning, especially those of Natalie Strynadka and Dr. Pam Werner. Dr. David Heeley who introduced me to the art of 2D gels, has always been willing to share his expertise in protein purification. Special thanks to Drs. Ruthven Lewis and David Mannock for hosting the Thanksgiving, Christmas and New Year celebrations during all these years. Dr. Mike James and members of his laboratory (especially Anita) have been most gracious for letting me participate in several of their lab events as an adopted member. I would also like to thank the generous hospitality provided by Dr. Marie Fraser and Natalie during the final stages of writing the thesis.

Finally, the financial assistance provided by the Department of Biochemistry in the form of a graduate teaching and research assistantship and a studentship from the Alberta Heritage Foundation for Medical Research as well as Dr. Kay during the writing of this dissertation is gratefully acknowledged.

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LISTS OF ABBREVIATIONS AND SYMBOLS

A(λ)	absorption as a function of wavelength
ATP	adenosine triphosphate
cAMP	adenosine 3', 5'- cyclic monophosphate
CaBP	calcium binding protein
CD	circular dichroism
CNBr	cyanogen bromide
CNS	central nervous system
Cys	cysteine
DEAE- / DE	diethylaminoethyl-
DPA	dipicolinic acid
DTNB	5, 5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothrietol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
$E_{1cm,\lambda}^{1\%}$	absorbance of a 1% protein solution in a 1 cm pathlength cell at the wavelength, λ
F	fluorescence intensity
f	fractional conformational change
GdnHCl	guanidine hydrochloride
HLH	helix - loop - helix
HPLC	high pressure liquid chromatography
ICaBP	intestinal calcium binding protein or Calbindin D_{9k}
I.D.	internal diameter
K_d	dissociation constant
kDa	kilodalton

MAPS	microtubule associated proteins
MES	2-(*N*-morpholino)ethanesulfonic acid
MOPS	3-morpholinopropanesulfonic acid
MLCK	myosin light chain kinase
M_r	relative molecular mass
$\overline{M}_w$	weight average molecular weight
NBS	N-Bromosuccinimide
NEF	neurite extension factor
^{1}H NMR	proton NMR
^{113}Cd NMR	cadmium NMR
OD	optical density
PAGE	polyacrylamide gel electrophoresis
pH	negative logarithm of the hydrogen ion activity
Phe	phenylalanine
p*K*	negative logarithm of the dissociation constant
PIA	*N*-(1-pyrenyl)iodoacetamide
PIPES	piperazine-*N*, *N'*-bis(2-ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
ppm	chemical shifts of resonances in NMR
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
TFP	trifluoperazine
Tn-C	troponin-C
TNM	tetranitromethane
TNS	2-toluidinylnaphthalene-6-sulfonate
Tris/HCl	tris(hydroxymethyl)aminomethane hydrochloride

Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet
W-7	*N*-(6-aminohexyl)5-chloro-1-naphthalene sulfonamide
ε	molar extinction coefficient
$\Delta\varepsilon$	molar difference extinction coefficient
ε_{max}	absorption maximum
Φ_F	quantum yield
λ_{ex}	excitation wavelength
λ_{max}	wavelength at which the fluorescence emission maximum occurs
$[\theta]_\lambda$	mean residue ellipticity at the wavelength, λ
$[Ca^{2+}]_i$	intracellular concentration of free calcium ion
τ	tau protein, one of the microtubule associated proteins
~	approximately

I. INTRODUCTION

A. Calcium in Biological Systems

1. Functions of calcium in biological systems

The functions of calcium in biological systems are not only diverse and multifaceted but also fundamental in nature (Campbell, 1983). Calcium, being a major constituent of bone and teeth, plays a structural role by providing rigidity to organisms. It also plays an indirect role in maintaining osmotic balance of cells through its role in integrity and permeability properties of biological membranes. At another level, calcium, by promoting cellular adhesion, stimulates intercellular communications that are vital for optimizing cellular functions by facilitating the exchange of ions and macromolecules through gap junctions. Calcium is also a cofactor for several extracellular degradative enzymes and proteins which require the ion for stability or maximal activity in both prokaryotes and eukaryotes. In this respect, the metal is involved in a host of activities associated with physiological functions in serum and other body fluids such as blood clotting, complement reactions involving antibodies and in digestion. However, the most significant biological function of calcium lies in its role as a ubiquitous intracellular mediator. Calcium, cyclic AMP (Rasmussen *et al.*, 1985) as well as the recently discovered diacylglycerol and inositol trisphosphate (Berridge, 1987), serve as second messengers for their respective extracellular signals, thereby coupling a diverse array of cellular responses to various external stimuli. Intracellular functions mediated by calcium include glycogenolysis, cyclic nucleotide and lipid metabolism, cellular motility as manifested by cytoplasmic streaming, cell shape changes, ciliary motion and muscle contraction, microtubule assembly and disassembly, secretion, phototransduction and gene expression. Since each of these activities must be regulated independently and occur simultaneously in different parts of the cell, the calcium signal must exhibit spatial as well as temporal resolution.

The ability of calcium to act as a second messenger resides in the fact that the resting intracellular concentration of free calcium ion, $[Ca^{2+}]_{i(rest)}$ or "background Ca^{2+}" is very low in eukaryotic cells (1×10^{-7} M to 3×10^{-7} M), compared to the extracellular free Ca^{2+} concentration of ~ 1×10^{-3} M (Ashley and Campbell, 1979; Blinks *et al.*, 1982). Upon the interaction of an extracellular messenger, which can be either chemical or electrical in nature, with its specific cell surface receptor, the concentration of cytosolic free calcium increases to 10^{-6} - 10^{-5}M (Rasmussen, 1981). Owing to the low intracellular Ca^{2+} background, a small increase in the absolute amount of free calcium ion in the cytosol results in amplification of the signal of the external stimuli by 1 to 10 or even 100 fold in some cells (Blaustein, 1985). The sharp increase in $[Ca^{2+}]_i$, which activates a wide variety of Ca^{2+} mediated physiological responses, can be initiated in 2 major ways: Ca^{2+} can be mobilized from intracellular storage sites associated with the endoplasmic reticulum (Berridge and Irvine, 1984). Alternatively, 1,4,5-inositol trisphosphate, generated from cleavage of phosphatidylinositol-4,5-bisphosphate, upon binding to its receptors, may lead to the release of Ca^{2+} into the cytoplasm from intracellular storage sites (Berridge, 1987). Upon cell activation, the plasma membrane becomes more permeable to Ca^{2+} (Borle, 1981), which then enters the cell via its steep electrochemical gradient through the voltage sensitive and receptor operated calcium channels (Miller, 1987). However, the peak rise in $[Ca^{2+}]_i$ is brief and soon falls to a level of 0.3 to 0.5 μM (Charesi *et al.*, 1983; Snowdowne and Borle, 1984; Feinstein *et al.*, 1983; O' Doherty *et al.*, 1980). Nevertheless, the slightly higher than basal value of $[Ca^{2+}]_i$ is sufficient to sustain cellular responses during the steady state phase of cell activation. The maintenance of the low level of free calcium is achieved by a complex system which involves cytosolic buffering proteins such as parvalbumin and calbindin D_{28k} and intracellular organelles such as the endoplasmic reticulum which act as temporary storage sites for Ca^{2+} until they are extruded from the cell by the ATP driven Ca^{2+} pump and the Na^+/Ca^{2+} exchanger in the plasma membrane (Blaustein, 1988). In addition to regulating the magnitude and the time course of the rise of $[Ca^{2+}]_i$, calcium buffering (removing) also serves to terminate the intracellular signal as well as determining the spatial profile of $[Ca^{2+}]_i$ (McBurney and Neering, 1987; Williams *et al.*, 1985).

Once the Ca^{2+} signal is generated, it is then transduced by a wide variety of calcium binding proteins, (CaBPs), which in turn transmit the signal to specific cellular compartments. Some of the proteins, by binding Ca^{2+} in the physiological range of 10^{-7} to 10^{-5} M in a reversible manner, act as sensors to mediate the triggering effects of Ca^{2+} (Levine and Williams, 1982). These include calmodulin, a ubiquitous protein present in eukaryotic cells. Hence, Ca^{2+} influx/efflux controls calmodulin activation which in turn activates a number of intracellular enzymes (Means, 1988; Wang *et al.*, 1985; Cheung, 1984; Klee *et al.*,1980). Furthermore, calmodulin through its interaction with the multifunctional Ca^{2+}/calmodulin dependent kinases (Schulman and Lou, 1989; Stull *et al.*, 1986), also provides a means of sustaining cellular responses long after the termination of the Ca^{2+} signal. In skeletal and cardiac muscle, troponin-C modulates the Ca^{2+} mediated contraction / relaxation cycle at the level of the thin filament through the complex of troponin and tropomyosin (Leavis and Gergely, 1984; Zot and Potter, 1987; Kay *et al.*, 1987). However, there are CaBPs that suppress the intracellular activities of Ca^{2+} either directly, as in the case of the Ca^{2+}ATPase in the plasma membrane which extrudes Ca^{2+} from the cytosol space (Penniston, 1983) or via a supramolecular structure in which the phosphatase, calcineurin, forms a Ca^{2+} dependent complex with calmodulin which can inhibit some of the calmodulin dependent enzymes (Cheung, 1988).

In addition to its role in mediating cellular responses, Ca^{2+} is also involved in modulating the intracellular level of another second messenger, cAMP. The enzymes catalyzing cAMP synthesis and degradation, adenylate cyclase and phosphodiesterase, are both activated by the Ca^{2+} calmodulin complex (Cheung, 1980). Furthermore, the activities of some of the calmodulin dependent enzymes, such as phosphorylase kinase and MLCK, are modulated by cAMP dependent kinase (Malencik and Fischer, 1982; Conti and

Adelstein, 1980). Thus, this illustrates that both cAMP and Ca^{2+} act as synarchic messengers in stimulus-response coupling (Rasmussen, 1981).

2. Role of calcium in nervous tissue

In the nervous system, neurons or nerve cells are responsible for intercellular communication which takes the form of electrical impulses being propagated along the neuron plasma membrane and via transmitters across the synaptic clefts. Ca^{2+} is intimately involved in both of these processes. Upon the depolarization of a membrane by an action potential, extracellular Ca^{2+} enters the cell through voltage sensitive Ca^{2+} channels. The influx of these ions carries the depolarizing charge across the membrane and contributes to electrical excitability such as pacemaker depolarization as well as generating a Ca^{2+} potential in some cells (Erulkar and Fine, 1979). Ca^{2+} also functions in the opening and closing (*i.e.* gating) of ionic channels that are specific for Na^+ and K^+. A rise in the $[Ca^{2+}]_i$ activates calcium sensitive K^+ channels (Meech, 1978) that are responsible for the repolarization of the cell membrane during the downward phase of the action potential. The importance of calcium in normal nerve excitability is seen when upon removal of extracellular Ca^{2+}, spontaneous firing of squid and frog nerves occurs (Brink *et al.*, 1946). The entry of Ca^{2+} into neurons also serves as a trigger for neurotransmitter release at nerve synapses. However, the molecular mechanism underlying this triggering is not known, though it has been suggested that the interaction of Ca^{2+} with its "trigger receptor" may influence the process of exocytosis (Smith and Augustine, 1988). In addition to its role in excitation-gating and excitation-secretion coupling, Ca^{2+} is also important in the maintenance of axoplasmic transport in nerves, thereby conveying nutrients, proteins and other synthesized compounds from their site of synthesis in the soma (cell body) into nerve fibres to maintain normal physiological functions (Ochs and Iqbal, 1982). Ca^{2+} is also required for neural outgrowth and retractions as well as growth cone motility in nervous tissues. The long term effects of changes in $[Ca^{2+}]_i$ lead to neuronal protein synthesis and gene expression which may be responsible for adaptive changes such as those associated with learning and memory (Morgan and Curran, 1988). Therefore, calcium in addition to its basic metabolic and 'housekeeping" roles also performs specialized functions in the nervous system. In order to understand the structure and function of the nervous system at the molecular level, it is important to characterize those proteins that are specific to nervous tissues through which the highly specialized functions are mediated.

B. S-100 Protein

1. Nervous tissue highly enriched in S-100 Protein

S-100 protein, originally discovered by Moore (1965), was thought to be a protein specific to the nervous system. The designation is based on its near complete solubility in 100% ammonium sulfate at neutral pH. S-100 protein has been detected with a constant immunological response in nervous tissues of vertebrates and invertebrates (Moore *et al.*, 1968) suggesting that its structure is highly conserved during phylogenesis (Moore, 1972). This has led to the suggestion that S-100 protein may serve an important role in the functioning of the nervous system. The recent mapping of the S-100β gene to human chromosome 21 (Allore *et al.*, 1987) has led to the suggestion that an increased gene dosage and over expression of S-100, which might result from the triplication of this chromosome in Down's syndrome, may have a role in the etiology of this disease.

The properties of the S-100 proteins have been documented in several reviews (Moore, 1975; Bock, 1978; Zomzely-Neurath, 1982; Donato, 1986; Mani and Kay, 1987a; Van Eldik, 1988). Essentially, S-100 is a water soluble, highly acidic protein with a molecular weight of 21,000 (Moore, 1965; Moore and McGregor, 1965; Dannies and Levine, 1969). It is found primarily in the cytoplasm of glial cells (Hyden and McEwen, 1966), representing up to 0.5% of the total soluble protein in bovine brain (Moore, 1965). In general, S-100 protein is concentrated in the cerebellum, followed by the cerebrum, brain stem and spinal cord (Moore *et al.*, 1968; Suzuki *et al.*, 1982; Kimura *et al.*, 1984), with the white matter having a higher concentration than the grey. The concentration of the protein varies from 10-20 μM depending on the region considered (Moore *et al.*, 1968). The S-100 protein appears late in the development of the nervous system, at a time when histological maturity is well advanced (Cicero *et al.*, 1970). In rats, the S-100 level in whole brain is low from birth until 10 days postnatally and then rises rapidly to a stable level at 90-120 days of age (Moore, 1969; Cicero, 1972).

2. Isoforms and subunit composition of the S-100 protein

During the earlier studies of S-100 protein, the heterogeneous nature of the protein was observed on its electrophoretic analysis using various buffer systems (McEwan and Hyden, 1966; Gombos *et al.*, 1971a,b). In 12.5% polyacrylamide gel, the S-100 protein migrates as a single polypeptide at or close to the dye front, with a subunit molecular weight of 10,000 (Calissano *et al.*, 1976), suggesting that the 21,000 dalton native protein consists of polypeptides intermolecularly linked. Dannies and Levine (1969) had purified a mixture of S-100 isoforms from bovine brain. Subsequently, upon separating the carboxymethylated protein into its respective subunits by gel filtration and anion exchange chromatography in the presence of denaturants, mobility measurements in SDS polyacrylamide gel gave a subunit molecular weight of 7,000, with reference to the mobilities of several protein standards. Sedimentation equilibrium studies also suggested a weight average molecular weight ($\overline{M}_w$) of 6,800 for one of the subunits, compared to a $\overline{M}_w$ of 21,300 for the unfractionated protein. Hence, Dannies and Levine (1969; 1971) concluded from their studies that the S-100 protein was composed of 3 subunits, each with a molecular weight of 7,000. Subsequently, Isobe and Okuyama, using improved purification procedures and from sequencing studies (Isobe and Okuyama, 1977; 1978; Isobe *et al.*, 1981; Isobe and Okuyama, 1981a,b) established that the S-100 protein in bovine brain is made up of 2 major isoforms, S-100a and S-100b. Both isoforms exist as dimers, with S-100b containing two β subunits, whilst S-100a is a heterodimer made up of an α and β chain, with a molecular weight of around 10,000 for each subunit. Both isoforms are highly acidic, with a pI of 4.2 and 4.5 for S-100a and S-100b, respectively

(Manabe *et al.*, 1982). Using high performance liquid chromatography, a third isoform, S-100a$_0$, consisting of $\alpha\alpha$ homodimer, which represents 2 - 4% of total S-100 protein, has also been identified (Isobe *et al.*, 1983).

Bovine brain contains the highest concentration of S-100, giving a yield of 200 - 250 mg/kg wet tissue (Moore, 1982; Masure *et al.*, 1984), and the 2 major isoforms, S-100a and S-100b occur in approximately equal concentration. However, S-100 protein has also been purified from rat brain which contains almost exclusively (90 - 95%) $\beta\beta$ homodimer (Matsuda *et al.*, 1983; Isobe *et al.*, 1983; Baudier *et al.*, 1985). In human brain, 80 - 96% of the total S-100 protein is S-100β (Baudier *et al.*, 1984; Jensen *et al.*, 1985) and the S-100 protein purified from bovine adipose tissue is also identical to S-100β (Marshak *et al.*, 1985). On the other hand, the S-100 protein that has been isolated from rat kidney (Semba *et al.*, 1987), and human cardiac and skeletal muscle (Haimoto and Kato, 1988), resembles the α-subunit from bovine brain. These studies, therefore, show that the presence of S-100 is not restricted to nervous tissue alone and it appears that there are species differences in the relative amounts of the various isoforms of S-100 protein.

3. Primary Structure of S-100 protein

The amino acid sequence of both the α and β subunit from bovine brain have been published (Isobe and Okuyama, 1978, 1981a,b). The α subunit is comprised of 93 residues, with a predicted molecular weight of 10,400 whilst the β subunit contains 91 residues, the molecular weight of which has been predicted to be 10,507. The sequences shown in Fig. 1, have been aligned according to maximum homology by introducing 2 gaps in the β subunit corresponding to positions 1 and 90 on the α subunit. A comparison of the primary structures of the 2 subunits revealed that they are highly homologous. Of the 93 residues, 54 are identical, which results in a 58% identity between the 2 subunits. In addition, there are 23 replacements that are compatible with single nucleotide base substitutions. The high degree of homology between the 2 subunits is more notable between residues 1-73 (with 63% homology) than the C-terminal region, i.e. residues 74-

```
       1       5         10        15        20        25        30        35        40        45
α:  b  G S E L E T A M E T L I N V F H A H S G K E G D K Y K L S K K E L K E L L Q T E L S G F L
β:  Ac S E L E K A V V A L I D V F H Q Y S G R E G D K H K L K K S E L K E L I N N E L S H F L
         o o o o * o * * * o o * o o o   * o o * o o o o * o o   o   o o o o o *   * o o o   o o

               50        55        60        65        70        75        80        85        90    93
α:     D A Q K D A D A V D K V M K E L[D E D G D G E V D F Q E]Y V V L V A A L T V A C N N F F W E N S
                                       |   |   |   |   |     |
β:     E E I K E Q E V V D K V M E T L[D S D G D G E C D F Q E]F M A F V A M I T T A C H E F F - E H E
                                       |   |   |   |   |     |
       * *   o *   * * o o o o o *   o o   o o o o o   o o o o * * * * o o   * o   o o *   o o   o *
                                       |   |   |   |   |     |
                                       x   y   z  -y  -x    -z
```

Figure 1 - A comparison of the amino acid sequences between the α and β-subunits of bovine brain S-100 protein (Isobe and Okuyama, 1981a). The two sequences are aligned by maximum homology. The sign (o) stands for identical residues in both sequences and (*) for a substitution compatible with a single base mutation, illustrating the extensive homology between the two subunits. The putative calcium binding site is bracketed, with x, y, z, -y, -x and -z representing the ligands that coordinate the calcium ion. Amino acid residues are identified by single letter notations, A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp and Y, Tyr. Ac refers to an acetyl group and b, a blocking group not identified.

93 (with 40% homology at this end) (Moore, 1988). Both subunits are characterized by a blocked N-terminus and a high content of acidic residues, representing about 30% of the total amino acids. In addition, both subunits contain a significant amount of aromatic amino acids, and in the case of the β subunit, a high Phe / Tyr ratio gives rise to its distinctive UV absorption spectrum (Mani and Kay, 1987a). The α-subunit is characterized by both a single cysteine and tryptophan residue located at positions 85 and 90, respectively. The β-subunit contains tyrosine and arginine residues at positions 17 and 20, respectively, which can be used to distinguish the α from the β-subunit, upon performing an amino acid analysis of the S-100 protein. In addition, the β-subunit also contains 5 histidines, compared to 2 in the α-chain. Histidines are known to coordinate Zn^{2+} ligands (Holmes and Matthews, 1981) and the fact that at physiological pH, both S-100a and S-100b bind Zn^{2+} with higher affinity than Ca^{2+} (Baudier *et al.*, 1986), suggests that these residues may have an important role in the Zn^{2+} mediated functions of S-100 that remain to be identified.

Along the polypeptide chains of both subunits, there are 4 clusters of chemically similar residues. At the N-terminus, there is a stretch of hydrophobic residues, from residues 4 - 15, followed by a cluster of basic amino acids at positions 21 - 34. Towards the C-terminal end, an acidic cluster is located between residues 60 - 73, followed by another hydrophobic region between residues 73 - 90, thereby, giving the appearance of a "dipole" on the two halves of the molecule. Furthermore, the N-terminal half of the molecule (residues 1 - 48) is almost neutral in charge (net charge of -1) in contrast to the C-terminal half (residues 49 - 93), with a net charge of -9 in the α-subunit. In the dimeric molecule, the 2 subunits appear to interact non-covalently since all 3 cysteines in S-100a (1 in α and 2 in β) were found to be available on titration with DTNB (Isobe and Okuyama, 1981a). It appears that the 2 subunits are held together by ionic bonds, which probably involve chemical groups that ionize below pH 3 (Morero and Weber, 1982) as well as hydrophobic forces resulting from the interaction of aromatic amino acid side chains that are buried inside the protein matrix (Baudier and Gerard, 1986).

The amino acid sequences of S-100 from rat and human are also available. The primary structure of human S-100 (80 - 96% S-100β) reveals a difference of 3 amino acids from bovine brain S-100β (Jensen *et al.*, 1985), and these changes are compatible with minimum single base changes in codon structures. In the case of rat S-100 (90 - 95%β), the amino acid sequence deduced from the nucleotide sequence shows rat S-100β to differ from its bovine counterpart in 4 positions (Kuwano *et al.*, 1984; Dunn *et al.*, 1987). Thus, while there appear to be species differences in the amount of S-100β in the brain, the primary structure of the β-chain is structurally conserved in mammals. This finding is in line with earlier observations of interspecies conservation of immunoreactive sites amongst S-100 protein using antisera prepared against the bovine brain protein (Levine and Moore, 1965; Manabe *et al.*, 1982). In the case of S-100α, less information is available regarding its structural conservation. Kuwano *et al.*,(1986) reported the isolation of a S-100α cDNA from bovine brain, which showed that apart from 1 residue, the predicted amino acid sequence derived from the nucleotide sequence is identical to the published primary structure.

4. Calcium binding sites predicted from the primary structure

Upon examination of the amino acid sequences of S-100α and S-100β, a calcium binding site has been predicted to exist on each of the subunits (Isobe and Okuyama 1978, 1981a). The calcium binding site has sequence homology with the EF hand type structure that was first observed in carp parvalbumin (Kretsinger and Nockolds, 1973). An EF hand contains certain invariant amino acids in a loop structure flanked by 2 approximately perpendicular α helices; the loop functions as the actual Ca^{2+} binding site (Kretsinger, 1980). Other Ca^{2+} binding proteins such as calmodulin (Watterson *et al.*, 1980), troponin-C (Van Eerd *et al.*, 1973; Collins *et al.*, 1977), calbindin D_{9k} also known as the intestinal calcium binding protein, ICaBP (Fullmer and Wasserman, 1981)) and carp parvalbumin (Coffee and Bradshaw, 1973) also contain this common helix-loop-helix motif of approximately 30 residues in length. Examination of the refined crystal structure of bovine brain calmodulin (Babu *et al.*, 1988), turkey skeletal troponin-C (Herzberg and James, 1988) and chicken Tn-C (Satyshur *et al.*, 1988) showed that the Ca^{2+} coordination geometry in the EF hand Ca^{2+} binding sites is pentagonal bipyramidal. According to the nomenclature derived from the original octahedral description of ligand geometry (Kretsinger and Nockolds, 1973), six residues at positions 1 (X), 3 (Y), 5 (Z), 7 (-Y), 9 (-X), and 12 (-Z) in the loop provide oxygen ligands to the Ca^{2+} ion. In all the proteins that contain EF hands, the residue at -Z is invariably a glutamate and contributes both side chain oxygens to metal ion coordination. This bidentate coordination at -Z, along with the oxygen ligands at Y, Z, -Y defines an approximately planar pentagonal arrangement that includes the central Ca^{2+} ion. The vector that joins the bipyramidal apices, occupied by an oxygen atom from an invariant aspartate at X to the opposite apex which, in general, is an oxygen atom from a water molecule (Herzberg and James, 1985), passes near to the Ca^{2+} ion and is approximately perpendicular to the pentagonal plane. With such a coordination, all 7 ligands are at an average of 2.4 Å from the Ca^{2+} ion (Strynadka and James, 1989). In Fig. 2, the sequence in the C-terminal region (residues 48 - 81) of the α and β subunits of S-100 is aligned with the canonical sequences proposed by Tufty and Kretsinger (1975) for predicting EF hand motifs in proteins. Of the 16 residues required for forming such a motif, 14 residues are found in the β-subunit. The seven oxygen ligands required for pentagonal bipyramid Ca^{2+} coordination appear to be provided by residues at positions: Asp 61(X), Asp 63(Y), Asp 65(Z), Glu 67(-Y), Asp 69(-X) and Glu 72(-Z) on the β-chain. The 2 residues that are inconsistent with the test sequence are Val 52β and Cys 68β. On the α-subunit, the Ca^{2+} ligands are identical to those found in the β subunit and the only mismatch with the test sequence is found at Ala 53α (Isobe and Okuyama, 1978). The C-terminal Ca^{2+} binding site in S-100 is very similar to those observed in calmodulin (sequence also shown in Fig. 2). In both cases, the calcium binding loop contains 12

Figure 2. A comparison between the test sequence proposed by Tufty and Kretsinger (1975) for predicting EF hands and those found in calmodulin (Watterson *et al.*, 1980) and the rearranged Ca^{2+} binding domains of calbindin 9K (Fullmer and Wasserman, 1981) and S-100 protein (Isobe and Okuyama, 1978, 1981a). The test sequence represents residues critical to the EF hand structure. In the test sequence, * indicates an oxygen containing residue (D,E,N,Q,S,T) and corresponds to the Ca^{2+} coordinating ligands, X, Y, Z, -Y, -X and -Z; L indicates a hydrophobic residue (L,V,I,F,M), I indicates a hydrophobic residue where isoleucine is preferred; G is glycine and an underscore indicates any amino acid may appear in that position. Insertions and deletions have been postulated to maximize homology between the sequences. Amino acids are identified by single letter code (as defined in Fig. 1) (adapted from Szebenyi *et al.*, 1981).

		Helix	Loop	Helix	Linker
Test Sequence	E	L - - L L - - L	* - * - * G - I * - - *	L - - L L - - L	
Bovine brain S-100α		S /\	Y /\		
	10 b G S E L E T A M E T	L I N V F H A H	G K E G D K K L S K K E	L K E L L Q T E	L S G F L D A Q K D A D
	A 53	V D K V M K E L	D E D G D G E V D F Q E	Y V V L V A A L	T V A C N N F F W E N S 93
Bovine brain S-100β		S /\	H /\		
	AcS E L E K A V A A	L I D V F H Q Y	G R E G D K K L K K S E	L K E L I N N E	L S H F L E E I K E Q E
	V 52	V D K V M E T L	D S D G D G E C D F Q E	F M A F V A M I	T [illegible] A C H E F F E H E 91
Calf Calbindin 9K		A /\	N /\		
	K S P E E 1	L K G I P E K Y	A K E G D P Q L S K E E	L K L L L Q T E	F P S L L K G P S 44
	T 45	L D E L F E E L	D K N G D G E V S F E E	F Q V L V K K I	S Q 75
Bovine brain calmodulin	E Q I A E. 7	F K E A F S L F	D K D G D G T I T T K E	L G T V M R S L	G Q N P T E A 46
	E	L Q D M I N E V	D A D G N G T I D F P E	F L T M M A R K	M K D T D S E E 83
	E	I R E A F R V F	D K D G N G Y I S A A E	L R H V M T N L	G E K L T D E Me_3 119
	E	V D E M I R E A	N I D G D G Q V N Y E E	F V Q M M T A K 148	

residues that are characteristic of EF hands. However, S-100 has the additional feature of a predominance of acidic ligands compared to calmodulin.

Based on the genealogical tree proposed by Goodman (1980), the S-100 protein has been suggested to be evolutionary related to calbindin D_{9K}, and that the two proteins were anciently separated from the other helix -loop - helix lineage as represented by calmodulin and troponin-C. The crystal structure of calbindin D_{9k} (Szebenyi *et al.*, 1981; Szebenyi and Moffat, 1986), revealed that in addition to an EF hand Ca^{2+} binding site at the C-terminal region, a pseudo variant site characterized by a 2 amino acid insertion is located at the N-terminus. As a result of the conformational change that has arisen in the longer unconventional loop, 4 main chain carbonyls, a water molecule and a carboxylate side chain coordinated Ca^{2+} in calbindin D_{9k}. Since the S-100 protein has been suggested to be evolutionary related to calbindin D_{9k}, Szebenyi *et al.* (1981) predicted that a pseudo EF hand site is also located in the N-terminal region of S-100 protein (residues 19 -32), with the 3 basic residues Lys- His - Lys in positions corresponding to Pro - Asn - Gln sequence in the I-II Ca^{2+} binding loop of the calbindin D_{9k}.

5. Prediction of secondary structure of S-100 protein

The three dimensional structure of the S-100 protein is not known despite a report on the crystallization of S-100b (Kretsinger *et al.*, 1980). Secondary structure analysis using the predictive algorithm of Chou and Fasman (1974) as well as a modified version (Dufton and Hider, 1977), suggested that both the α and β subunits have a similar distribution of α helices, β sheets and turns. As illustrated in the diagram in Appendix I, along the polypeptide chain of the α-subunit, an α helix is predicted to exist between residues 1 - 16, followed by a β turn at residues 18 - 21, and another helix at residues 22 - 40. In the C-terminal half of the molecule, an α helix at residues 45 - 60 precedes the Ca^{2+} binding loop. This is followed by a turn which begins at the first Ca^{2+} coordinating ligand at position 62. Another helix at residues 68 - 73 exits from this helix-loop-helix motif which typifies proteins containing EF hand type Ca^{2+} binding sites and this is immediately followed by a β sheet structure extending from residues 74 - 90. The β-subunit is predicted to exhibit a similar distribution of secondary structure as found in the α-chain, with the exception that a long helix extends from residues 83 - 90 on the β-chain.

6. Family of S-100 like proteins

One the most recent developments in the field of S-100 protein research is the report of proteins or cDNA structures with homology to S-100 (Kligman and Hilt, 1988). Each member of the S-100 protein family is approximately 100 residues long, and contains a conserved hydrophobic domain as well as a Ca^{2+} binding domain similar to the EF hand prototype at both ends of the molecule. Members of this family include: (1) a growth factor inducible gene coding for a protein, calcyclin or 2A9, with 40% identity to S-100β (Calebretta *et al.*, 1986); (2) cDNAs 18A2 and PEL 98, which contain nucleotide sequences that code for proteins with 47% identity to S-100 (Jackson-Grusby *et al.*, 1987; Goto *et al.*, 1988); (3) cDNAs 42A and 42C that are induced upon treatment of PC 12 cells with nerve growth factor and which code for proteins with 46% and 35% identity respectively to S-100β (Masiakowski and Shooter, 1988); (4) a gene coding for protein p9Ka expressed in rat mammary myoepithelial cells with 46% identity to S-100β (Barraclough *et al.*, 1987); (5) proteins MRP-8 and MRP-14 with 34% and 37% identity, respectively, to S-100β that are found in infiltrate macrophages in chronic inflammatory lesions (Odink *et al.*, 1987); (6) cystic fibrosis serum antigen, CF Ag, with 31% identity with S-100β (Dorin *et al.*, 1987); (7) the p11 subunit or the regulatory chain of p36 complex (calpactin I), a substrate for a viral growth factor associated tyrosine specific protein kinase with 43 and 33 identically placed residues when compared with S-100α and S-100β, respectively (Gerke and Weber, 1985; Saris *et al.*, 1987), and (8) a protein, PEP-19, isolated from rat cerebellar extract with sequence homology to S-100α (Ziai *et al.*, 1986). These proteins together with calbindin D_{9k}, with 32% homology to bovine S-100 (Kuwano *et al.*, 1984), indicate that there is a family of structurally related S-100 like proteins. Apart from S-100 protein, the ability to bind Ca^{2+} has only been demonstrated with CF Ag, MRP-8, MRP-14 and calbindin D_{9k}. The functions of some of the members of the S-100 protein family may be involved in cell cycle progression and cell differentiation. In this respect, it is interesting to note that the areas of greatest divergence in these proteins are found in the C-terminal region as well as a hinge region separating the 2 EF hands, which suggests that this region may have a role in conferring the specificity of the target proteins that interact with each of these S-100 proteins (Kligman and Hilt, 1988).

C. Metal ion binding properties of S-100 protein

1. Ca^{2+} binding properties

The Ca^{2+} binding properties of a crude mixture of S-100 (Calissano *et al.*, 1969; 1974; Morero and Weber, 1982) as well as single isoforms from bovine brain (Mani *et al.*, 1982; Mani and Kay, 1983a; Mani *et al.*, 1983; Baudier and Gerard, 1983; Umekawa *et al.*, 1983; Masure *et al.*, 1984; Baudier *et al.*, 1986; Baudier and Gerard, 1986; Baudier and Cole, 1989) have been investigated. In addition, S-100 protein from rat brain (Baudier *et al.*, 1985; Moore, 1988) and human brain (Baudier *et al.*, 1984) were also examined. The binding of Ca^{2+} to S-100 is pH dependent. At pH 7.5, one Ca^{2+} binding site exists on each subunit with an apparent dissociation constant, K_d, of 1.2×10^{-4} M and 2×10^{-4} M for bovine brain S-100a and S-100b, respectively (Mani *et al.*, 1983; Mani and Kay,1983a). At pH 8.5, S-100 binds 2 Ca^{2+}/ monomer; in addition to the presence of a low affinity site with a K_d value of 10^{-4} M, a high affinity site with an apparent K_d of 5×10^{-5} M and 6×10^{-5} M are also found on S-100a and S-100b, respectively. Direct Ca^{2+} binding studies at pH 8.3, using continuous flow dialysis also confirmed the existence of 4 Ca^{2+} binding sites/ dimer on each of the S-100 isoforms with K_d values in the range of 20 - 50 μM and 200 - 500 μM (Baudier *et al.*, 1986). In the brain, the concentration of intracellular free Ca^{2+}, at rest, has been reported to be approximately 0.35 μM (Bachelard *et al.*, 1988); hence, this raises the question as to whether S-100 can bind Ca^{2+} under physiological conditions. Both the α and β-subunits have identical ligands in the C-terminal EF hand Ca^{2+} binding site, which might account for some of the similar Ca^{2+} binding properties of various isoforms of S-100 protein. Irrespective of the fact that both the S-100 protein and calbindin D9k share a similar arrangement of 1 variant and 1 EF hand Ca^{2+} binding site, the S-100 protein binds Ca^{2+} with lower affinity than calbindin D_{9k}, which exhibits a K_d value of ~ 10^{-6} to 10^{-8} M (Shelling and Sykes, 1985). Szebenyi *et al.*, (1981) suggested that the lower affinity could be due to the presence of excess positively charged residues at the N-terminal Ca^{2+} binding site of the S-100 protein (+5 in the loop), which would to some extent deter Ca^{2+} approach to the C-terminal site. In addition, the conformational change made in order to accommodate the longer Ca^{2+} binding loop at the N-terminus, might also affect the Ca^{2+} affinity in the other half of the molecule. Another theory to explain the lower affinity of S-100 for Ca^{2+} is related to the large number and the positioning of acidic residues in the C-terminal EF hand site. Reid and Hodges (1980) observed that most of the Ca^{2+} binding loops in EF hands that have been sequenced contained acidic residues in 4 of the 6 coordinating positions, suggesting that this is the most stable arrangement of anionic charges in a metal-ion protein complex. They further postulated that the Ca^{2+} binding loop having the highest metal-ion binding affinity would have the four acidic ligands situated on the ± X and ± Z axes, as depicted by the octahedral coordination geometry (Kretsinger and Nockolds, 1973). The pairing of acid residues would allow the negatively charged residues to approach the cation from opposite sides, hence, maximizing the approach to cation while minimizing repulsion of like charges. In the case of S-100 protein, both the α and β subunits contain 6 acidic ligands in the C-terminal Ca^{2+} binding loop. Furthermore, these ligands occupy ±X, ±Y and ±Z axes, an arrangement that has been predicted to give highly unfavourable dentate-dentate repulsion. Hence, this might account for the low affinity of S-100 protein for this metal ion.

Upon binding Ca^{2+}, the S-100 protein undergoes a conformational change resulting in a loss in the α-helical content (Mani *et al.*, 1983; Mani and Kay, 1983a). Earlier studies also indicated that the conformational change induced by Ca^{2+} binding resulted in increased reactivity of 2 out of the 3 sulfhydryls in a mixture of S-100 proteins, towards the thiol reagent, DTNB (Calissano *et al.*, 1969, 1974). Moreover, these sulfhydryls have been implicated in contributing to the observed electrophoretic heterogeneity of the S-100 protein in non-denaturating gels (Rusca and Calissano, 1970; Calissano *et al.*, 1976). A study with S-100a protein that had been modified with a thiol specific fluorescence probe, N-(1-pyrenyl)iodoacetamide, revealed the labelled cysteine at position 84β, to move to a more polar environment in the presence of Ca^{2+} (Mani and Kay, 1985a). On the other hand, the labelling of Cys 84β in S-100b with the same fluorophore, indicated that the probe became less accessible in the presence of Ca^{2+}, compared to the absence of metal ions (Mani and Kay, 1985b). Hence these studies suggested that Ca^{2+} binding to S-100 protein induced a conformational change affecting the C-terminus where these sulfhydryls are situated.

Ca^{2+} binding to S-100 is antagonized by monovalent cations, with the order of effectiveness being $K^{+} > Na^{+} > Li^{+}$ (Calissano *et al.*, 1969; Mani and Kay, 1984). In the presence of KCl, S-100 affinity for Ca^{2+} decreased; for example, at pH 7.5, in S-100b, the apparent K_d is lowered from 2×10^{-4} M to 8×10^{-4} M (Mani *et al.*, 1983a). The addition of K^{+} to the Ca^{2+} saturated protein reversed most of the spectral changes induced by Ca^{2+} (Mani *et al.*, 1983, Baudier *et al.*, 1986). Mg^{2+}, another cation found intracellularly, when added to S-100 protein had no effect on the conformation (Mani and Kay, 1983a; Baudier *et al.*, 1986) but there are conflicting reports as to whether Mg^{2+} exerts an antagonistic effect towards Ca^{2+} binding to S-100 (Baudier *et al.*, 1986; Matsuda, 1988).

Biologists have often used calcium analogs to substitute for calcium in biological activity as well as to function as reporter groups from the Ca^{2+} binding site. In this respect, members of the lanthanide family which exhibit a wide variety of useful spectroscopic and magnetic properties, render them valuable in probing the metal ion binding environment of Ca^{2+} binding proteins (Horrocks, 1972; Dockter, 1983). Moreover, the overlap in the ionic radii of Ca^{2+} and lanthanides enables the amorphous replacement by the latter without significantly distorting the conformation of the protein.

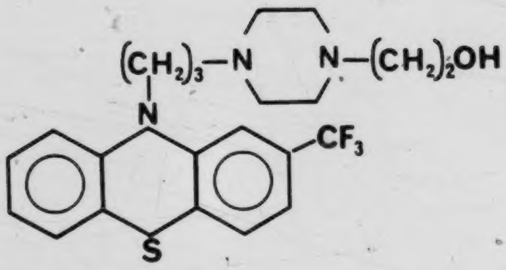
The fact that Tb^{3+} possesses a larger charge to volume ratio than Ca^{2+}, resulted in tighter binding of Tb^{3+} to proteins. Since terbium has luminescence properties which are sensitive to its environment, it appears to be the most widely used Ca^{2+} analog amongst the lanthanides. In the case of the S-100 protein, S-100a has been shown to bind 2 moles Tb^{3+}/mole of protein at pH 6.6. Compared with Ca^{2+}, Tb^{3+} binds tighter with an apparent K_d value having an upper limit of 3.5×10^{-6} M (Mani and Kay, 1987b). Spectroscopic studies revealed the protein to undergo a conformational change upon binding Tb^{3+}, resulting in a decrease in the α helical content as well as perturbation of the environment of the single tryptophan and three tyrosines, similar to that observed with Ca^{2+}. Hence, this suggests that Tb^{3+} can be used as a Ca^{2+} analog in the investigation of the functions of S-100 protein in future studies.

2. Zn^{2+} binding properties

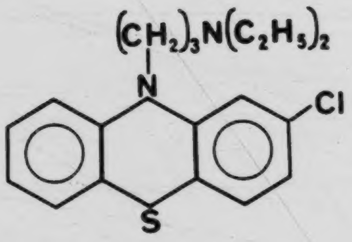
Recently, it has been discovered that the S-100 protein also binds Zn^{2+} with K_d values of 10^{-6} M - 10^{-8} M (Baudier and Gerard, 1983; Baudier *et al.* 1986; Mani and Kay, 1985a, 1985b). Unlike Ca^{2+}, the binding of Zn^{2+} is not pH dependent (Mani and Kay, 1983b). In the whole brain of normal mice, the total Zn^{2+} concentration has been reported to be around 270 - 280 μM in wet tissue (Chung and Johnson, 1983). This is similar to that found in the hippocampus (Donaldson *et al.*, 1972; Ebadi *et al.*, 1981), which is known to contain a high content of Zn^{2+}, especially in mossy fibre nerve terminals, in which the Zn^{2+} concentration has been estimated to be as high as 448 μM (Frederickson *et al.*, 1983). Hence, it is likely that S-100 can be saturated with Zn^{2+} under physiological conditions and may have a role in mediating the ill defined functions of Zn^{2+} in the brain. Furthermore, Zn^{2+} binding to S-100 protein induces conformational changes different from those of S-100a (Baudier *et al.*, 1982a; Mani and Kay, 1983b). The different Zn^{2+} binding properties of S-100a and S-100b may be related to differences in the number of histidines and cysteines present on each of the subunits, since these are the most common residues for Zn^{2+} coordination in enzymes and other proteins (Fersht, 1977).

D. Drug binding and Hydrophobic properties of S-100 protein

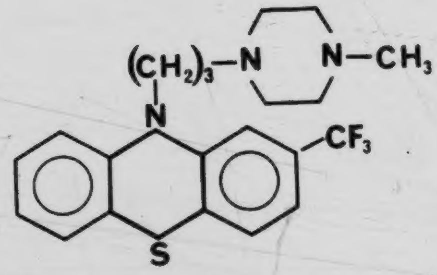
It is thought that a common denominator for the processing of the Ca^{2+} signal (i.e. interaction with targets) by high affinity CaBPs, is the exposure of hydrophobic sites upon Ca^{2+} complexation, although additional mechanisms which have yet to be discovered, may also exist (Carafoli, 1987). In the case of calmodulin, its action on several Ca^{2+} dependent enzymes is inhibited by phenothiazine antipsychotic drugs, which bind to calmodulin in a Ca^{2+} dependent manner involving both hydrophobic as well as ionic interactions with one of the piperazine nitrogens which possesses a positive charge at physiological pH (Prozialeck and Weiss, 1982). The S-100 protein has also been reported to interact with several of the antipsychotic compounds, the structures of which are illustrated in Fig. 3. In general, these compounds are characterized by the presence of a hydrophobic phenothiazine ring and an amino side chain several atoms away from the ring. Subsequently, this has led to the development of Ca^{2+} dependent affinity chromatography on a fluphenazine column (Masure *et al.*, 1984), as well as a 2-choloro-10-(3-aminopropyl) phenothiazine Sepharose column (Marshak *et al.*, 1981), to purify S-100 protein. On the other hand, Donato (1984a) reported that S-100 protein exhibited a similar affinity for chlorpromazine in the absence or presence of Ca^{2+}, suggesting that in some instances, the interaction of S-100 with antipsychotic drugs may not be strictly Ca^{2+} dependent. The S-100 protein is also known to bind to W-7 (or N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide), a calmodulin antagonist, which is structurally related to the phenothiazines to some extent (Hidaka *et al.*, 1981). Ca^{2+} dependent affinity chromatography on W-7 epoxy activated Sepharose 6B column, has allowed the purification of bovine brain S-100 from calmodulin (Endo *et al.*, 1981) as well as S-100 protein from bovine adipose tissue (Hidaka *et al.*,



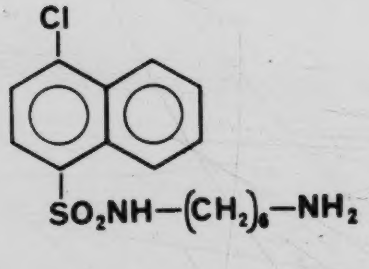
Fluphenazine



2-Chloro-10-(3-aminopropyl)
Phenothiazine



Trifluoperazine



N-(6-aminohexyl)5-chloro-1-
naphthalene sulfonamide (W-7)

Fig. 3 The structures of some of the hydrophobic compounds that bind to S-100 protein. The first three belong to the phenothiazine class of antipsychotic drugs.

1983). Furthermore, the 2 major isoforms of S-100 proteins, S-100a and S-100b, can also be separated on this column using a stepwise Ca^{2+} EGTA buffer (Umekawa *et al.*, 1983).The ability of S-100 protein to bind to these immobilized drug ligands in the presence of Ca^{2+} appears to correlate at least partly with their hydrophobicity, similar to that observed with calmodulin (Laporte *et al.*, 1980; Klevit *et al.*, 1981). Ca^{2+} binding to S-100a protein has been shown to induce a conformational change, which resulted in the hydrophobic fluorescence probe, 2-p-toluidinylnaphthalene-6-sulfonate (TNS), that was bound to the protein to move to a less polar environment (Baudier and Gerard, 1983). On the other hand, Ca^{2+} did not appear to affect the hydrophobic domains on S-100b protein. The S-100b protein has been reported to exhibit Ca^{2+} dependent binding of trifluoperazine (TFP), another member of the phenothiazine family. However, NMR studies revealed that the TFP - S100b exchange rate was different from that observed with calmodulin, suggesting that the TFP binding site(s) on S-100b is structurally different from its binding sites on calmodulin (Pingerelli *et al.*, 1989).

A number of neuropeptides and amphipathic cytoactive peptides have been known to inhibit the activation of calmodulin dependent enzymes, and to compete with them for binding to calmodulin *in vitro* (Malencik and Anderson, 1986). As a result, they have been used as models in studies of calmodulin binding sites that are specific for target enzymes. Amongst this group, S-100 has been reported to bind to melittin immobilized onto a Sepharose column (Kincaid and Coulson, 1985). Moreover, at pH 7.5, in the absence and presence of Ca^{2+}, S-100b and melittin formed a complex with molar ratios up to 2:1, and a dissociation constant of interaction estimated to be in the micromolar range (Baudier *et al.*, 1987). In addition, the latter group suggested that the conformational change induced in S-100b upon interacting with melittin, resulted in an increase in the affinity of S-100b for Ca^{2+}. Furthermore, the similarities in the melittin binding properties of S-100 with those observed in calmodulin has led to the postulation that the melittin binding site on S-100 might be structurally related to the corresponding domain on calmodulin (Baudier *et al.*, 1987).

E. Distribution of the S-100 protein

1. Cellular and subcellular distribution

Studies on the tissue distribution of S-100 protein may provide an insight into the potential function(s) of this protein. In the central nervous system, S-100 is found primarily in the glial cells and appears to be diffusely distributed throughout the cytoplasm, including the cell body and extensions, and associated with glial filaments. Whilst some investigators have reported localization of S-100 in the nucleus of glial cells (Hyden and McEwan, 1966; Sviridov *et al.*, 1972; Cocchia, 1981; Michetti *et al.*, 1974), others have not confirmed this finding (Tabuchi and Kirsch, 1975; Ghandour *et al.*, 1981). The inconsistency suggested in these earlier studies is most likely due to the fact that polyclonal antisera were raised against S-100 protein from bovine brain, which contained both S-100α and S-100β. Therefore, it is not known whether the observed localization patterns were due to S-100α and/or S-100β. With the development of antibodies monospecific to each of the subunits, recent studies have shown that S-100β is located primarily in the astrocytes, oligodendrocytes and ependymal cells while S-100α is located in neurons and astrocytes. Furthermore, the distribution appears to be different at the intracellular level, where the S-100α showed a granular arrangement in contrast to S-100β which is found diffusely throughout the cytoplasm (Isobe *et al.*, 1984a).

S-100 protein has also been localized in the peripheral nervous system. Earlier studies indicated S-100 to be present in Schwann cells of myelinated and unmyelinated peripheral nerves as well as in satellite cells of the sensory and autonomic ganglia (Stefansson *et al.*, 1982a). Subsequent studies using S-100α and S-100β specific antibodies showed that S-100β is localized in the Schwann cells and satellite cells, whereas S-100α is found in neurons of the peripheral nerves (Isobe *et al.*, 1984a). In addition, the staining pattern of S-100α and S-100β is similar to that observed in the central nervous system.

Studies conducted with human neoplastic tissues also revealed a distribution of S-100α and S-100β parallel to those observed in normal tissues (Takahashi *et al.*, 1984; Isobe *et al.*, 1984b). Subsequently, S-100 protein has been used as a marker for various types of tumors (Springall *et al*, 1983; Cocchia *et al.*, 1983) and to diagnose and classify tumors of neural origin (Kahn *at al.*, 1983).

In addition to polyclonal antibodies, the production of monoclonal antibodies that recognized unfractionated S-100 or either the α or β subunit has been reported (reviewed in Donato, 1986). One of these, A4, prepared from unfractionated bovine brain S-100, reacted with brain extracts from several mammalian and one avian species (Marks *et al.*, 1983). This suggests that the epitope common to both the α and β subunits is present in S-100 from different vertebrate species, a strong indicator of conservation of S-100 protein structure during evolution.

Despite the original suggestion that S-100 was thought to be a brain specific protein, various studies have demonstrated its presence in a variety of non-nervous tissues. These include Langerhans cells and melanocytes of the skin (Cocchia *et al.*, 1981); adipocytes (Michetti *et al.*, 1983); chondrocytes in the cartilage (Stefansson *et al*, 1982b); folliculostellate cells of the pituitary (Nakajima *et al.*, 1980); macrophages and interdigitating cells of human lymph nodes (Ushiki *et al.*, 1984) and renal tubules of kidney (Molin *et al.*, 1985). These studies demonstrated that S-100 cannot be regarded as a protein specific to nervous tissues.

2. Quantitation of the S-100 protein

The availability of S-100α and S-100β specific antibodies has led to the development of a sensitive enzyme immunoassay to quantitate the distribution of the three S-100 isoforms in various tissues from different mammalian sources. Measurements performed with bovine brain showed that the relative concentration of S-100a_0, S-100a and

S-100b are 2, 21 and 77%, respectively, in the cerebellar cortex (Kimura *et al.*, 1984), confirming the earlier finding that S-100a and S-100b are the predominant isoforms in the bovine brain (Isobe *et al.*, 1983). Studies performed with human tissues showed a high concentration of S-100α and S-100β in the brain, which amount to 0.3 μg/mg and 3 μg/mg of soluble protein, respectively. Surprisingly, both heart and skeletal muscle are found to contain a significant amount of S-100α (1-2 μg/mg) (Kato and Kimura, 1985). A similar pattern of S-100α in varying degrees of excess are also observed in the heart and skeletal muscle from bovine, rat and mouse sources (Kato *et al.*, 1986). Further studies with mouse skeletal muscle revealed that slow twitch fibres contain almost exclusively S-100a$_0$ and that its concentration is three times higher than that in fast twitch muscle (Haimoto and Kato, 1987).

At the ultrastructural level in skeletal muscle, S-100α is associated with polysomes, sarcoplasmic reticulum, mitochondria, as well as in the thick and thin filaments (Haimoto and Kato, 1987). In the case of human cardiac muscle, which contains twice the amount of S-100a$_0$ compared to skeletal muscle, it is localized in the nuclei and cytoplasm of myocardial cells and regions of the intercalated disc (Haimoto and Kato, 1988). Furthermore, the S-100α that has been purified from skeletal and cardiac muscle showed an amino acid composition similar to bovine brain S-100α (Haimoto and Kato, 1988; Donato *et al.*, 1989). The physiological significance of the S-100 protein in muscles is not known though it appears not to be involved in the contractile process.

3. Membrane bound S-100 protein

Though soluble for the most part a small fraction, 2 - 15% of the total S-100 in the brain is associated with the particulate fraction of cells (Haglid and Stavrou, 1973; Rusca *et al.*, 1972; Donato *et al.*, 1975). The particulate associated S-100 resists extraction with EDTA and high salt (1.0 M NaCl) and can be solubilized by n-pentanol (Haglid and Stavrou, 1973) or non-ionic detergents such as Triton X-100 (Rusca, *et al.*, 1972; Donato *et al.*, 1975). This suggests that some of the S-100 protein interacts strongly with membrane components. Donato (1977) has shown that synaptosomal preparations bound unfractionated ^{125}I S-100 and that this binding activity can be solubilized by Triton X-100. The interaction is specific and saturable with respect to S-100 concentration, suggesting the membrane contains a "receptor" for S-100. In addition to synaptosomal binding sites detected on the plasma membrane and synaptic vesicles (Donato, 1982), S-100 binding sites have also been found in isolated brain nuclei (Donato and Michetti, 1981). ^{125}I S-100 has been shown to concentrate in the nuclear membrane and nucleolus (Michetti and Donato, 1981). S-100 protein purified from isolated brain nuclei represents 0.55% of soluble S-100 in the cytosol and occurs as free S-100, labile bound S-100 found in the deoxyribonucleoprotein fraction and stable bound S-100 in the nucleolar fraction (Michetti *et al.*, 1974).

Immunocytochemical studies and indirect immunofluorescence microscopy (Hyden and Ronnback, 1975; Haglid *et al.*, 1977; Cocchia, 1981) have demonstrated that in adult brain, S-100 is detected in the membranes of the outer mitochondria, endoplasmic reticulum, Golgi apparatus, nuclear membrane, and plasma membrane. Subsequent studies have demonstrated that the cytoplasmic and membrane bound S-100 proteins from bovine and rat brains are identical (Donato *et al.*, 1986a).

F. Biological Function(s) of the S-100 protein

Despite numerous efforts, very little is known about the function of S-100 protein apart from its metal-ion binding properties and that it exists mainly in the cytoplasm of the glial cells. With respect to the distribution of S-100 in the brain, it is interesting to note that calmodulin, another abundant Ca^{2+} binding protein, (up to 400 mg/kg wet tissue) is found primarily in the neurons, distributed throughout the perikarya, somata and dendrites (Woods *et al.*, 1980; Tabuchi *et al.*, 1984). The observed reverse cellular distribution of calmodulin and S-100 protein in primate brain has led to the suggestion that calmodulin might be replaced in its role as an intracellular Ca^{2+} modulator by S-100 in glial cells

(Tabuchi *et al.*, 1984). More recently, there is increasing evidence to suggest that S-100 may have extracellular functions. In studying the function(s) of the S-100 protein, 2 approaches have been followed so far: (1) examination of S-100 interaction with isolated cellular components *in vitro* and (2) the isolation, characterization and identification of unknown S-100 target proteins. Despite these indirect approaches, useful information has been gathered to shed some light on the possible functions of the S-100 protein described below.

1. Regulation of membrane permeability

The identification of a membrane bound S-100 protein suggests that this subpopulation of S-100 might have a specialized function. Membranes are known to contain ionic channels which conduct current, thus providing the basis for action potential. These channels are normally impermeable to cations thereby maintaining a concentration gradient across the membrane, and thus generating the driving force for their electrical excitability. It has been suggested that S-100 upon interacting with artificial membranes alters their permeability to cations. The leakage of $^{86}Rb^+$ from, as well as the incorporation of $^{45}Ca^{2+}$ into, phosphatidylserine /phosphatidylcholine liposomes is greatly enhanced by S-100 in the presence of Ca^{2+} (Calissano and Bangham, 1971).

The purification of membrane bound S-100 from synaptosomes from cerebral cortex (Donato *et al.*, 1975) raised the question as to whether S-100 has another function associated with its presence in nerve endings. Influx of Ca^{2+} through the Ca^{2+} channels at the nerve synapses led to a generation of a Ca^{2+} action potential and also served as a trigger for neurotransmitter release. It has been reported that exogenous S-100, though ineffective on its own, potentiated the effects of the Ca^{2+} ionophore, A23187, on the release of putative neurotransmitters from rat brain synaptosomes (Gallo *et al.*, 1982). Hence, this has led to the suggestion that the role of S-100 in the membrane of nerve endings might be that of facilitating the function of an endogenous voltage dependent ionophore.

2. Modulation of phosphorylation and dephosphorylation

Ca^{2+} is known to regulate a wide range of biological effects through phosphorylation of effector proteins in the nervous system (Walaas and Nairn, 1985). Since S-100 has been found in the nucleus, the effect of S-100 on protein kinase activities in nuclei has been examined. Perumal (1976) reported an increase in total protein phosphorylation by brain nuclear kinases in the presence of S-100, a phenomenon subsequently demonstrated to be specific to the brain (Perumal and Rapport, 1978). In addition, S-100 stimulated the activity of a brain protein kinase (Qi *et al.*, 1984) as well as the phosphorylation of its endogenous protein substrate (Qi and Kuo, 1984) in a Ca^{2+} independent manner. The effects of S-100 protein are not restricted to stimulation of kinases activities; it also inhibits the phosphorylation of a 87 kDa protein by protein kinase-C (Kligman and Patel, 1986). Moreover, S-100 also affects the activity of retroviral tyrosine specific kinase by inhibiting the phosphorylation of the p36 subunit of calpactin I complex by retroviral gene products $p130^{frs}$ and $p60^{src}$ in the absence of Ca^{2+} (Hagiawara *et al.*, 1988). Hence, S-100 may regulate neural functions via stimulation or inhibition of phosphorylation by a wide variety of protein kinases. Besides kinases, S-100 also stimulated the activities of phosphoprotein phosphatases (Kuo *et al.*, 1986).

3. Regulation of assembly and disassembly of microtubules

Microtubules are one of the three major filamentous networks that comprise the cytoskeleton of eukaryotic cells. It is thought that their dynamic interplay with various cellular components is instrumental in motility, morphogenesis and maintenance of cell shape. Since S-100 protein has been observed to be associated with some of the microtubule structures (Michetti and Cocchia, 1982), the effect of S-100 on microtubule assembly and disassembly *in vitro* has been extensively investigated. S-100 protein has been reported to potentiate the Ca^{2+} induced disassembly of brain microtubules *in vitro*, at mM Ca^{2+} concentration more effectively than calmodulin (Baudier *et al.*, 1982b). This has led to the suggestion that S-100 exerts its effect by increasing the calcium sensitivity of microtubules (Donato, 1985). Subsequent studies have suggested a direct interaction between S-100 and tubulin (Endo and Hidaka, 1983; Fujii *et al.*, 1986)). Analysis of kinetic data (Donato, 1984b) and ultrastructural studies (Donato *et al.*, 1986b) suggested that S-100 by interacting and sequestering tubulin, interferes with the nucleation of new microtubules and elongation of existing microtubules during the assembly process (Donato, 1987; Donato *et al.*, 1987). In addition to interacting with tubulin, S-100 is also reported to interact with microtubule associated tau protein (τ) (Baudier *et al.*, 1987). Moreover, it has been demonstrated that Ca^{2+} stimulated the covalent complex formation of S-100b with τ (Baudier and Cole, 1988), and resulted in inhibiting the phosphorylation of τ by protein kinase-C and Ca^{2+}/calmodulin dependent kinase (Baudier *et al.*, 1987). In addition, the various isoforms of S-100 protein exhibit different effects on microtubules in the presence of Zn^{2+}. At low Zn^{2+}/S-100b molar ratio, S-100b was observed to induce disassembly and inhibit microtubule formation, whilst S-100a had no effect on the assembly process (Denium *et al.*, 1983). On the other hand, Donato *et al.* (1985) reported that in 0.1 to 1 mM free Zn^{2+}, both S-100a and S-100b exerted a similar effect in inhibiting assembly, whilst S-100a$_0$ was without effect. Furthermore, the suggestion that S-100 could play a role in organizing the cytoskeletal structure was accentuated by a recent finding which revealed that the synthesis of S-100β mRNA in a glioma cell line required the structural integrity of the microtubular network (Dunn *et al.*, 1987).

4. Interaction with target protein(s)

A second approach which has been useful in studying S-100 function is the detection and identification of its target proteins i.e. S-100 binding proteins. Gopalakrishna *et al.*, (1985) detected S-100 binding proteins from bovine brain extract which bind to S-100 specifically in a Ca^{2+} dependent manner, as well as others that bind in the absence of Ca^{2+}. Since S-100 is a calcium binding protein, it is not surprising to find that some proteins that bind to S-100 also bind to calmodulin and these include phosphorylase b, phosphorylase kinase, myosin (Burgess *et al.*, 1984), neuromodulin, also known as p43 (Baudier *et al.*, 1989) and caldesmon (Skripnikova and Gusev, 1989). With caldesmon, the Ca^{2+} dependent interaction with S-100 prevents the former from binding to actin filaments, thereby relieving its inhibitory effect on actomyosin Mg^{2+} ATPase activity (Fujii, *et al.*, 1990). Using a gel overlay technique, a protein from a rat brain extract was detected to bind to ^{125}I-S-100β, and subsequently shown to be related to the enzyme, fructose-1,6-bisphosphate aldolase (Zimmer and Van Eldik, 1986). Moreover, biochemical studies suggested that S-100 stimulated the *in vitro* activity of the brain specific isoform of this enzyme in a Ca^{2+} dependent manner. In a detailed study comparing the distribution of S-100α, S-100β and calmodulin binding proteins in various rat tissues, it was observed that each tissue possessed its own complement of S-100 binding proteins (Zimmer and Van Eldik, 1987), suggesting that S-100 may have tissue specific functions. In addition, the calmodulin binding protein profile did not resemble that of S-100, implying in this case, that S-100 and calmodulin interact with different molecular targets.

5. Regulation of cell cycle progression / differentiation and gene expression

Ca^{2+} is known to be an important regulator of cell proliferation (Whitfield *et al.*, 1976), and is also implicated as a key component in regulating cell mitosis (Ratan and Shelanski, 1986). Means and Rasmussen (1988) have suggested that calmodulin is involved in regulating cell cycle progression since its increased expression at the G_1/S boundary is necessary for cells to initiate and proceed through DNA synthesis. The fact that S-100β is expressed differently during the cell cycle, with peak synthesis coinciding with that of calmodulin, suggests that S-100 might have a role in mediating cell cycle progression (Fan, 1982). The cell cycle and cell differentiation associated expression of the S-100 like protein family has been reviewed by Kligman and Hilt (1988). Since cyclic changes in the phosphorylation of specific proteins is an established feature of the cell cycle, it is speculated that S-100, by mediating the phosphorylation of its target proteins, may be responsible for its demonstrated effect in cell cycle progression and cell type differentiation. The localization of S-100 protein in the nucleus and its presence in isolated nuclei has led to the suggestion that S-100 may function in gene regulation via interactions with proteins and enzymes which regulate gene expression. Further studies have also shown that S-100 protein stimulated brain RNA polymerase I activity (Michetti *et al.*, 1976).

6. Extracellular functions of S-100 protein

In addition to a role for S-100 as an intracellular Ca^{2+} mediator, evidence is accumulating that some form of S-100 may be secreted and have an extracellular function. The detection of S-100 protein in the extracellular space of the brain has led to the speculation that the secretion of the protein into the extracellular space may provide a means of transporting S-100 from its site of synthesis in the glial cells to neurons where it exerts its action (Shashoua *et al.*, 1984).

Recently, it has been reported that bovine brain extracts (Kligman and Marshak, 1985) or conditioned media of mouse neuroblastoma cells (Kligman and Hsieh, 1987) stimulate neurite outgrowth from chicken embryo cerebral cortical neurons. This neurite extension factor (NEF) activity has been partially purified and appears to be identical to a disulfide linked S-100β dimer. The suggestion that S-100 may act as a neurotrophic factor in the CNS is further supported by the demonstration that *in vitro* expressed recombinant S-100β has neurite extension activity and enhances the survival of neurons in cell culture (Van Eldik *et al.*, 1988; Winningham-Major *et al.*, 1989). The neurotrophic activity of S-100β appeared to require the presence of disulfide bond(s) , since treatment with reducing agents abolished the NEF activity. Moreover, genetically engineered mutants lacking either cysteine 68 or 84 showed a significant decrease in ability to stimulate neurite extension.

The potential NEF activity for S-100β is consistent with previous studies that suggested a role of S-100 in nervous system development. For instance, the increase in the amount of S-100 coincides with periods of brain development when neuronal processes are elongating (Cicero *et al.*, 1970). In addition, the levels of translatable mRNA for S-100β increase during similar periods of development in rat brain (Matsuda *et al.*, 1983). Hence, these studies raise the possibility that during development of the nervous system, S-100β might be secreted from glial cells and act in a paracrine fashion to stimulate neurite outgrowth.

G. Aims of the thesis

In the past our laboratory has concentrated on elucidating the molecular mechanism of Ca^{2+}, through the interaction with its receptor, troponin-C, in the regulation of muscle contraction. Tn-C belongs to a family of intracellular proteins that share a common Ca^{2+} binding motif consisting of a helix-loop-helix (HLH) that has been subsequently described as an EF hand (Kretsinger, 1980). The S-100 protein is evolutionarily related to Tn-C and other members of the HLH family (Isobe and Okuyama, 1978; 1981a). In response to the Ca^{2+} signal, these proteins trigger different responses or interact with different target proteins. Our ultimate goal is to establish the subtle molecular differences that exist between these proteins which enable them to elicit responses in different cells or tissues. In the case of the S-100 protein, though the 2 major isoforms, S-100a and S-100b, have been characterized physico-chemically, no detailed study has been performed on the S-100 isoform containing the αα homodimer. Since Ca^{2+} and Zn^{2+} binding to S-100a and S-100b exert different effects, it was anticipated that by characterizing the physico-chemical and chemical properties of the α-subunit and comparison with those observed in the β-subunit, a better understanding of the observed differences in the metal ion binding properties of the principal isoforms could be obtained.

To this end, a procedure for isolating the α-subunit from its parent molecule, S-100a protein, has been developed. Both hydrodynamic and spectroscopic approaches were employed to elucidate the physico-chemical properties as well as the effects of metal ion binding on the α-subunit. Since metal ion binding to the α-subunit induced the protein to undergo a conformational change, two fluorescent probes which interacted specifically with sulfhydryl groups and hydrophobic domains, were introduced into the α-subunit to serve as reporter groups in order to study the effect of metal ion binding on the local environment of these chromophores in the protein. Furthermore, since Ca^{2+} also perturbed the microenvironment of several aromatic chromophores, attempts were made to study the contribution of these residues to the overall conformational change observed upon the addition of this metal ion. To this end, the single tryptophan and one or both tyrosines in the α-subunit were modified with N-bromosuccinimide and tetranitromethane treatment, respectively. A comparison of the metal ion binding properties of the chemically modified and the native α-subunit was undertaken. Finally, the effect of S-100 protein on the *in vitro* activity of the brain specific isoform of fructose,1-6-bisphosphate aldolase was examined in order to address the question as to whether the S-100 protein could function as an *in vitro* calcium modulated effector protein.

II. EXPERIMENTAL PROCEDURES

A. Polyacrylamide Gel Electrophoresis

In these studies, both disc and slab SDS PAGE were employed for the analyses of protein homogeneity, as well as for the estimation of apparent molecular weights. Disc electrophoresis was performed according to the method of Weber and Osborn, (1969) in 6 x 100 mm tubes. The final gel concentration was 12.5% acrylamide, 0.375% bisacrylamide and 0.1% SDS. A buffer system consisting of 50 mM sodium phosphate, pH 7.0 was used and the gels were electrophoresed at 3 mA/tube for 4 hr. In the case of slab SDS PAGE, the buffer system of Laemmli (1970) (i.e., Tris-glycine) was used.

SDS gel electrophoresis was also performed in the presence of urea following the procedures of Swank and Munkres (1971), with the modification that the concentration of urea was 6 M instead of 8 M. The final gel concentration was 12.5% acrylamide at a polyacrylamide/bisacrylamide ratio of 30:1. Electrophoresis was carried out in 6 x 100 mm tubes at 3 mA/tube for 5 hrs.

In order to resolve the S-100a from S-100b protein, electrophoresis was carried out under native conditions, according to the procedure of Davies (1964). A buffer system containing Tris-glycine, pH 8.6, and 0.1% thioglycollic acid was used. The final gel concentration was 20% at a polyacrylamide/bisacrylamide ratio of 40:1. Disc electrophoresis was performed at 3 mA/tube for 10 hr. A slightly modified version of non-denaturing PAGE was also used to illustrate the different mobilities of calcium binding proteins in the presence and absence of Ca^{2+}. In this case, a more porous gel (final gel concentration of 12.5% acrylamide at a polyacrylamide/bisacrylamide ratio of 150:1), containing either 1 mM $CaCl_2$ or 1 mM EDTA was used. Disc electrophoresis was carried out at 3 mA/tube for 3 hrs and in the absence of thioglycollic acid.

Protein samples for SDS PAGE were treated with 5% 2-mercaptoethanol and 2% SDS (and in 6 M urea for urea/SDS/PAGE) followed by heat treatment in a boiling water bath for 5 mins. When electrophoresis was performed under non-denaturing conditions, the SDS and heat treatment steps were omitted. Bromophenol blue was added to the sample as a dye marker. After electrophoresis, the gels were stained with Coomassie Blue and destained in several changes of 10% methanol and 10% acetic acid.

B. Amino Acid Analysis

Samples were dissolved in 6 M HCl containing 0.1% phenol and hydrolyzed in an evacuated sealed tube at 110°C for 24 hrs. The hydrolysate was then evaporated to dryness over NaOH pellets in a vacuum dessicator followed by analysis on a Durrum D-500 amino acid analyzer (Dionex Corp). Half cystine was determined as cysteic acid after oxidation in performic acid as described by Glazer *et al.* (1975). Tryptophan was determined spectrophotometrically by the method of Bencze and Schmid (1957). On some occasions, the concentrations of protein samples were determined using amino acid analysis by comparing peak areas of eluted amino acids with those obtained from standard sets of amino acids (8 nmoles each). When calculating the concentration of the α-subunit from amino acid analysis, a subunit molecular weight of 10,400 was used (Isobe and Okuyama, 1981a).

C. Preparation of Protein Samples

Protein samples utilized for spectroscopic studies were made metal free by dialysis against 8 M urea, 0.1 M Tris/HCl, 10 mM DTT, pH 8.0. Chelex was also added to the solvent. After 6-8 hours, the denaturant was removed by dialyzing the protein extensively against 4 to 5 changes of chelex treated 0.1 M Tris/HCl, pH 7.5 or 8.4, and in the presence of 15 mM 2-mercaptoethanol. Prior to the optical studies, the samples were clarified by centrifugation in a Beckman Eppendorf microcentrifuge. In most cases, the concentration of the α-subunit was established by absorption measurements, using a molar extinction coefficient of 8300 $M^{-1}cm^{-1}$ at 278 nm (Masure *et al.*, 1984), and a molecular weight of

10,400 per α-chain (Isobe and Okuyama, 1981a). In the case of S-100a protein, a $E_{1cm,278}^{1\%}$ value of 5.4 was used (Mani and Kay, 1983a). Only plasticware was used for sample preparation and handling to eliminate contamination by metal ions.

D. Metal Ion Binding Assays

1. Calcium binding assay. The calcium binding assays were performed according to a modification of the method used to study Ca^{2+} binding to turkey Tn-C (McCubbin *et al.*, 1986). Essentially, an ultrafiltration device from Amicon Corp., the Micropartition System (MPS-1), which utilized the principle of the equilibrium nature of ultrafiltration partition, was used to perform rapid flow dialysis (Blatt *et al.*, 1968) as a means of monitoring Ca^{2+} binding to the α-subunit. Basically, an excess of $CaCl_2$ of known concentration was added to an aliquot of apoprotein ($1.2 - 2.3 \times 10^{-4}$ M) that had been previously dialyzed against 0.1 M Tris/HCl, 15 mM 2-mercaptoethanol, pH 7.5 or 8.4. After incubation at room temperature for 30 min, each of the samples was introduced into the top compartment of the ultrafiltration units and centrifuged in a Beckman model L centrifuge equipped with a 30K rotor at 4,500 rpm for 20 mins at 20°C. This process partitioned the free Ca^{2+} from the Ca^{2+} bound to the protein which was retained by an ultrafiltration membrane with a molecular weight cut off of 10,000 kDa. The concentration of the free Ca^{2+} (i.e., the solution centrifuged across the membrane) was determined using the metallochromic indicator, Arsenazo III (Aldrich Chemicals) according to the procedures of Thomas (1982). Essentially, this was done by monitoring in a spectrophotometer, the Ca^{2+} induced change in the absorbance at 650 nm, of a 30 μM indicator solution (in 100 mM Pipes, pH 7.1). Polystyrene cells of 1 cm pathlength were used. The absorbance readings were calibrated against a standard curve (known amount of Ca^{2+} added versus ΔA_{650}). A minimum of 4 assays were performed on aliquots from each ultrafiltrate sample. In order to correct for nonspecific adsorption of Ca^{2+} by the membrane, measurements were also performed on aliquots of ultrafiltrate obtained from solvent blanks that contained the same amount of Ca^{2+} as those added to the protein samples. Hence, from the amount of free Ca^{2+} found in the ultrafiltrate, along with the total amount of Ca^{2+} added and the amount of protein used, it was possible to calculate the amount of Ca^{2+} bound per mole of subunit. In the studies where the effect of K^+ on Ca^{2+} binding was examined, KCl (90 mM) was added to the apoprotein 15 mins prior to Ca^{2+} addition.

2. **Zinc binding assay.** The binding stoichiometry of Zn^{2+} to the α-subunit was also examined by the ultrafiltration technique as described for Ca^{2+}. An excess of $ZnSO_4$ of known concentration was added to the apoprotein which had been dialyzed against 100 mM Tris/HCl, 15 mM 2-mercaptoethanol, pH 7.5. After incubating for 30 mins at room temperature, the samples were centrifuged in order to partition the free Zn^{2+} from the Zn^{2+} bound to the protein. The concentration of the free Zn^{2+} in the ultrafiltrate was determined using the metallochromic indicator, 2,4-Bis-[N,N'-di-(carboxylmethyl)aminomethyl] fluorescein (Sigma Chemicals) according to Wallach and Steck (1963). Although Zn^{2+} can form a complex with fluorescein between pH 6.5 - 8.5, owing to the high pH used in the assay, no significant change in the fluorescence intensity was observed upon the formation of a complex. However, because of the inherent stability of the Zn^{2+} chelate, Zn^{2+}, even at low concentration can displace Co^{2+} from its complex with fluorescein, resulting in an increase in the fluorescence intensity. Hence, the concentration of Zn^{2+} (in μM level) can be determined by monitoring the increase in fluorescence intensity at 509 nm, of a solution of fluorescein (32 μM in 0.1 M Tris/HCl, pH 7.5), in the presence of 24 μM $CoCl_2$, upon the addition of Zn^{2+}. The excitation wavelength was set at 486 nm in these measurements. A minimum of 2 measurements were taken for each ultrafiltrate sample and these were calibrated against a standard curve. Measurements were also performed with ultrafiltrate samples obtained from solvent blanks containing Zn^{2+} at concentrations identical to those present in the protein samples. The latter procedure was used to correct for nonspecific adsorption of Zn^{2+} by the membrane.

3. Terbium binding assay. The binding stoichiometry of Tb^{3+} to the α-subunit was determined using the ultrafiltration technique as described above. The apo-protein used in the assay had been dialyzed against 50 mM Pipes, 5 mM 2-mercaptoethanol, pH 6.5. A buffer of lower pH was used to prevent the formation of hydroxyl complexes of Tb^{3+} which are insoluble. A 1.5 fold excess of Tb^{3+} (stock concentration of 1 mM) was added per α-subunit and incubated at room temperature for 30 min before partitioning the bound Tb^{3+} from the free by centrifugation. The concentration of the free Tb^{3+} in the ultrafiltrate was determined using the metallochromic indicator, dipicolinic acid (DPA), in a spectrofluorimeter according to the methodology described by Barela and Sherry (1976). DPA is essentially an aromatic chelator of Tb^{3+}, with high affinity for the metal ion (Grenthe, 1961). Primarily, it serves as an energy acceptor and subsequently donates this energy to the bound Tb^{3+} in the Tb^{3+} $(DPA)_3^{3-}$ complex, resulting in a 10^4 fold enhancement in the fluorescence intensity at 543 nm (F_{543}) (Barela and Sherry, 1976). The concentration of free Tb^{3+} was determined by monitoring F_{543} in a 1 cm quartz cuvette, containing 2 ml of 292 μM DPA (Sigma Chemicals), dissolved in 50 mM sodium acetate and 300 mM NaCl, pH 5.1. The fluorescence intensity of the ultrafiltrate was calibrated against a standard curve (known Tb^{3+} concentration versus F_{543} readings). Measurements were also performed with ultrafiltrate samples obtained from solvent blanks containing Tb^{3+} at concentrations identical to those present in the protein samples to correct for nonspecific adsorption by membranes.

E. Optical methods

1. Absorption spectroscopy. Routine absorption measurements, first derivative spectra and difference spectra were recorded employing a Perkin-Elmer Lambda 5 double beam spectrophotometer. Dialysates used to establish baselines were filtered through 0.22 micron Millipore filters.

Difference spectra were recorded over the wavelength range of 250-350 nm, with 1 cm pathlength cells. The instrument was operated with a slit width of 2 mm and at a scan rate of 7.5 nm/min. The spectra were recorded at ambient temperature, with the aid of a Lauda thermoregulator connected to both sample compartments. In order to study the effect of a perturbant upon a protein sample, a baseline was first generated with both cells containing equal volumes of protein solutions of identical composition. Then a small volume of concentrated perturbant (either $CaCl_2$, $ZnSO_4$, KCl or $MgCl_2$) was added to the sample cell and an equal volume of chelex treated water was added to the reference cell. Corrections were made for dilutions before the spectra were plotted as molar difference absorption coefficient, $\Delta\varepsilon$, with units of $M^{-1}cm^{-1}$.

2. Spectrophotometric titrations. Spectrophotometric titrations of the α-subunit were performed in 50 mM NaCl, 5 mM Tris/HCl, pH 7.5, 1 mM DTT and in the presence of either 1 mM Ca^{2+} or 1 mM EDTA. Samples (2 ml) were titrated with NaOH in a constantly stirred vessel. After addition of base, an aliquot was removed and the absorbance measured at 295 nm in a Perkin Elmer Lambda 5 spectrophotometer. The pH of the solution was determined with the aid of a Radiometer 64 pH meter. Two pH readings were taken after each addition of base, one prior to and one after the absorbance was measured. The 2 pH readings were then averaged. The 2 sets of readings obtained in the absence and presence of Ca^{2+} were normalized as percentage change in molar extinction coefficient at 295 nm. The experimental data were then fitted to the Henderson-Hasselbach equation:

$$pK = pH - \log \frac{[\alpha]}{[1 - \alpha]} \qquad [1]$$

where:

pK is the negative logarithm of the apparent dissociation constant of the phenolic group

α is the fraction of the total number of ionized chromophores

The theoretical curves were calculated based on the assumption that both of the tyrosines in the α-subunit contributed equally to the observed change in $\Delta\varepsilon_{295}$.

3. Circular dichroism. Circular dichroism measurements were performed using a Jasco J-500C spectropolarimeter fitted with a DPN-500 data processor as described previously (Mani and Kay, 1983a). The instrument was calibrated with an aqueous solution of d-10-camphor-sulfonic acid and pantoyl lactone. Constant nitrogen flushing of the instrument was employed. Spectra were recorded at 25°C with the aid of a Lauda thermoregulator connected to the sample compartment.

The circular dichroic spectrum of a protein arises from a differential absorption of left and right circularly polarized light, and at any given wavelength, it can be defined as $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$, where $\Delta\varepsilon$ is the circular dichroism and ε_L and ε_R are absorption coefficients of left and right circularly polarized light, respectively. As the emergent light is elliptically polarized, it is conventional to express it in terms of mean residue ellipticity, $[\theta]_\lambda$, defined by the equation:

$$[\theta]_\lambda = \frac{\mathrm{MRW} \cdot \theta_{obs}}{100 \cdot \ell \cdot c} \qquad [2]$$

where

MRW is the mean residue weight (in the case of α-subunit, this is taken as 110.929 and for aldolase,107.573,based on the amino acid compositions)

θ_{obs} is the observed ellipticity value at the wavelength of interest,

ℓ is the pathlength of the cell in dm, and

c is the concentration of the protein in g cm^{-3}.

The units of $[\theta]_\lambda$ are degrees.cm^2.decimole^{-1}.

The CD spectra of proteins in the far UV region (i.e., 190-230 nm) depend primarily on their secondary structures. In a sample, the ellipticity at any given wavelength can be expressed as the sum of the contribution from α-helix, β-sheet and random coil:

$$[\theta]_\lambda = f_H\,[\theta]_{H,\lambda} + f_\beta\,[\theta]_{\beta,\lambda} + f_R\,[\theta]_{R,\lambda} \qquad [3]$$

f_H, f_β, f_R are the predicted fractions of each structure, and $[\theta]_{H,\lambda}$, $[\theta]_{\beta,\lambda}$ and $[\theta]_{R,\lambda}$ are the values that represent the known intensity of each structural type at a given wavelength. The relative intensity is based on the CD spectra of 5 proteins that have had their structures previously determined by X-ray crystallography (Chen *et al.*, 1974).

The α-helical content of the α-subunit was estimated by solving equation [3] using the mean ellipticity values observed at 210, 215 and 220 nm of the CD spectrum as well as the reference protein values provided by Chen *et al.* (1974). Provencher and Glöckner (1981) developed another method whereby the CD spectrum is analyzed as a linear combination of CD spectra of 16 proteins with known structures. Furthermore, the method also includes a fourth structural entity, the β-turn. A computer program, Contin I (from European Molecular Biology Laboratory), for applying this method was also used to analyze changes in the secondary structural content of the α-subunit after addition of metal ions.

The near UV CD spectrum (i.e., 250-320 nm) of most proteins derives mainly from the aromatic residues, tryptophan, tyrosine and phenylalanine. They possess a low level of CD when existing as free amino acids in solution. However, the Cotton effects can be greatly enhanced if these chromophores are found nearby asymmetric structures such as α-helix and β-form and also upon interaction with other aromatic residues (Strickland, 1974).

CD measurements in the far and near UV region can provide information on the secondary structure and local environment of aromatic residues, respectively. Since proteins often undergo a conformational change upon binding metal ions, by monitoring the CD signal as a function of the concentration of metal ions, one can determine an apparent dissociation constant from the titration curve. The latter is defined as the concentration of metal ion required to induce a 50% change in the conformational parameter being monitored. CD titration data were normalized using the following expression:

$$f = \frac{[\theta]^{i}_{278}\text{nm} - [\theta]^{obs}_{278}\text{nm}}{[\theta]^{i}_{278}\text{nm} - [\theta]^{f}_{278}\text{nm}} \qquad [4]$$

where:

$[\theta]^{obs}_{278}$nm	is the ellipticity value at 278 nm at a given Ca^{2+} concentration,
$[\theta]^{i}_{278}$nm	is the initial ellipticity value at 278 nm before the addition of Ca^{2+},

and,

$[\theta]^{f}_{278}$nm	is the final ellipticity value at 278 nm after saturation of the protein with Ca^{2+}

The fractional conformational change, (f), was plotted versus the concentration of the total amount of Ca^{2+} added.

4. **Fluorescence spectroscopy.** Fluorescence excitation and emission spectra were obtained on a Perkin-Elmer model MPF-44B spectrofluorimeter operated in the ratio mode. The jacketed cell compartment was maintained at 25°C and in some cases, 20°C by a circulating constant-temperature water bath. The A_{278} of protein samples was maintained below 0.1 to avoid inner filter effects. All spectra were corrected for solvent baseline.

F. Denaturation Studies

Denaturation studies of the α-subunit were examined by using guanidine hydrochloride (GdnHCl) and heat as denaturants. In the thermal denaturation studies, the buffer system employed was 25 mM Pipes, 100 mM NaCl, 1 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.1. In the guanidine hydrochloride studies, a similar buffer system containing 25 mM MOPS, pH 7.1 and 2 mM DTT was used. GdnHCl was also dissolved in the same buffer and aliquots of stock solution (6 M) added to the protein in the absence and presence of 1 mM Ca^{2+}. The samples were allowed to equilibrate for 2 hrs at room temperature before obtaining CD measurements. The unfolding of the protein was monitored by following the decrease in $[\theta]_{221}$ values as a function of temperature or the concentration of GdnHCl added. Furthermore, the denaturation data obtained in the GdnHCl studies were normalized according to the following expression:

$$f = \frac{[\theta]^{i}_{221}\mathrm{nm} - [\theta]^{obs}_{221}\mathrm{nm}}{[\theta]^{i}_{221}\mathrm{nm} - [\theta]^{f}_{221}\mathrm{nm}} \qquad [5]$$

where:

$[\theta]^{i}_{221}\mathrm{nm}$	is the initial ellipticity value at 221 nm, before the addition of any GdnHCl,
$[\theta]^{f}_{221}\mathrm{nm}$	is the ellipticity value at 221 nm of the unfolded protein after addition of GdnHCl, and
$[\theta]^{obs}_{221}\mathrm{nm}$	is the ellipticity value at 221 nm at a given GdnHCl concentration.

The denaturation curves were obtained by plotting the fractional conformational change, f, as a function of the concentration of GdnHCl added. In the case of thermal denaturation, the mean ellipticity values at 221 nm were plotted as a function of the temperature.

G. Ultracentrifuge Methods

Ultracentrifugation studies were performed at 20°C in a Beckman Spinco Model E analytical ultracentrifuge. The instruments were equipped with a photoelectric scanner as well as a Raleigh interference optical system.

1. **Sedimentation equilibrium experiments.** Weight average molecular weights of the α-subunit were determined using conventional low speed sedimentation equilibrium techniques (Richards *et al.*, 1968). Double sector charcoal-filled Epon centerpieces (12 mm) were employed. Depending on the protein concentrations, measurements were made using either Raleigh interference optics or the UV absorption optics of the photoelectric scanner . The data were recorded either on photographic plates or chart paper of a photoelectric scanner accessory. Protein samples were dialyzed for at least 48 hrs. at 4°C to ensure complete equilibration (Casassa and Eisenberg, 1964). The initial protein concentrations (C_0) of these samples were determined using synthetic boundary runs (Richards *et al.*, 1968). Equilibrium measurements were performed with 0.1 ml of protein solution, using rotor speeds chosen from graphical data (Chervenka, 1968). Runs were assumed to be at equilibrium when no further changes in fringe position or pen tracing occurred.

The apparent weight average molecular weight, $\overline{M}_w$ of a homogeneous protein, determined by such an experiment, is given by:

$$\bar{M}w(r) = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \cdot \frac{d\ln c(r)}{dr^2} \qquad [6]$$

where:

R is the universal gas constant,

T is the experimental temperature in °K,

$\bar{v}$ is the partial specific volume of the protein, where, in the presence of Ca^{2+}, Zn^{2+} or EDTA, these values are 0.74, 0.728 and 0.71, respectively.

ρ is the solvent density,

ω is the angular velocity, and

c is the protein concentration at distance r, from the axis of rotation.

Where Raleigh interference optics were employed, protein concentrations at the meniscus, C_m, were calculated according to:

$$C_m = C_o - \frac{r_b^2(C_b - C_m) - \int_{cm}^{cb} r^2 dc}{r_b^2 - r_m^2} \qquad [7]$$

where:

C_o is the initial protein concentration,

C_m is the protein concentration at the meniscus,

r_m is the distance from the meniscus to the axis of rotation,

r_b is the distance from the cell bottom to the axis of rotation,

C_b is the protein concentration at the cell bottom, and,

C is the protein concentration at any distance, r, from the axis of rotation.

A direct measure of protein concentration in terms of fringes, Y, at any given value of r, was determined by adding the fringe displacement at point r to the concentration at the meniscus (expressed in fringes).

If the photoelectric scanner was employed, the absorption value, A, at any point along the cell provided a direct measure of the protein concentration.

A plot of the natural logarithm of the protein concentration (expressed in fringes, Y, or absorbance units, A) versus r^2 yielded a straight line, the slope of which d $\ln c/dr^2$, was used to determine the weight average molecular weight (using equation [6]) at any position along the cell.

To facilitate these calculations, computer programs, written by Dr. W.T. Wolodko (1974) were employed.

2. Protein concentration determination. The concentration of α-subunit that was labelled with fluorescent probe was determined using ultracentrifugal synthetic boundary runs with Raleigh interference optics (Babul and Stellwagen, 1969). A measurement of the number of fringes crossed on the photographic plate using a microcomparator, was related to the protein concentration using the average refractive index increment of 4.1 fringes/mg/ml determined for proteins by Babul and Stellwagen (1969).

H. Labelling of S-100 Protein with Fluorescent Probes

1. **Reaction of S-100 protein with TNS.** The fluorescent probe, 2-toluidino-naphthalene-6-sulfonic acid TNS, (sodium salt, from Molecular Probes) was dissolved in ultrapure water (Milli-Q instrument) prior to use. The concentration of the stock TNS solution (7-8 mM) was determined from the absorbance at 317 nm, assuming a molar extinction coefficient of 1.89×10^4 M (McClure and Edelman, 1966). Aliquots of stock TNS were added to the S-100 protein (5×10^{-6} M) that had been dialyzed against 0.1 M Tris/HCl, pH 7.5, in the absence and presence of metal ions, to give a final reagent to protein ratio of 3:1. Fluorescence measurements of TNS labelled S-100a protein and α-subunit were performed using an excitation wavelength of 345 nm and a filter which cut off fluorescence with wavelength emission shorter than 390 nm. Fluorescence titrations were performed by adding aliquots of stock TNS solution to the protein in the presence of 1 mM Ca^{2+}.

2. **Preparation of acrylodan labelled α-subunit and S-100a protein.** The fluorescent probe, 6-Acryloyl-2-dimethylaminonaphthalene (Acrylodan) (from Molecular Probes), was used without further purification. The S-100 protein was labelled according to the procedures of Prendergast *et al.* (1983). Although the mechanism of the reaction of Acrylodan with the sulfhydryl group is not known, a model reaction has been proposed and shown in Appendix II. Stock solutions of Acrylodan ($5 - 10 \times 10^{-3}$ M) were made up in dimethyl formamide. Aliquots of stock Acrylodan solution were added to the α-subunit (10^{-4} - 10^{-5} M) dissolved in 50 mM Mops, 1 mM Ca^{2+}, pH 7.0, to give a fluorophore:sulfhydryl group ratio of 4:1. In the case of S-100a protein, the labelling was performed under identical conditions except that 1 mM EDTA was present in the buffer system. To prevent denaturing the protein, the final concentration of the dimethyl

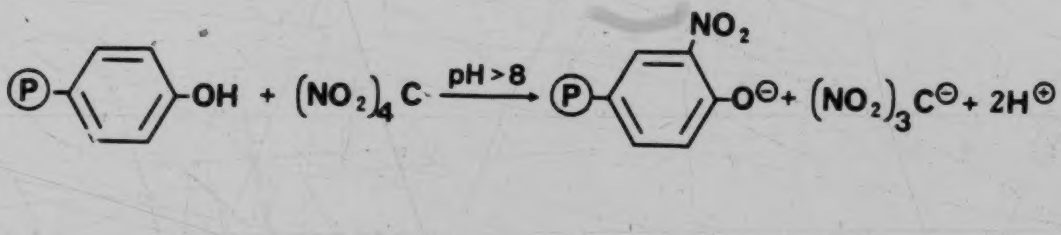
formamide added was kept below 4%. The reaction was allowed to proceed at 4°C for 12-14 hrs and then stopped by adding excess 2-mercaptoethanol. After labelling, the protein was subjected to clarification by centrifugation. The protein was initially dialyzed against 50 mM Mops, pH 7.0, 4 mM EDTA and thereafter extensive dialysis against chelex treated buffer containing 0.1 M Tris/HCl, pH 7.5. The concentration of the labelled protein was established in the ultracentrifuge as described in Section IIG. The concentration of the probe bound to the protein was determined from the absorbance at 360 nm using a molar extinction coefficient of 12900 $M^{-1}cm^{-1}$ (Prendergast *et al.*, 1983). Fluorescence measurements of the Acrylodan labelled S-100 protein were performed using an excitation wavelength of 380 nm and a filter which cut off fluorescence emission with wavelength shorter than 390 nm.

I. Protein Modification Techniques

1. Reaction of S-100 protein with DTNB. The thiol specific reagent, 5,5'-dithiobis(2-nitrobenzoic acid), DTNB (Ellman, 1969) (Sigma Chemicals) was used to study the reactivity of the sulfhydryl groups in S-100 protein under varying conditions. Stock DTNB (1×10^{-2} M) was made up in 50 mM $NaPO_4$, pH 7.2 and added to the α-subunit or the S-100a protein in 50 mM Mops, pH 7.0, in the absence or presence of metal ions. The ratio of reagent: sulfhydryl used was 10:1. Prior to the reaction, the protein was made metal free as described previously in Section IIC, except that no reducing agent was present in the buffer during the final dialysis. The reactivity of the sulfhydryl group(s) in the unfolded protein was also examined by performing the reaction in 6 M GdnHCl, 0.1 M Mops, pH 7.0. The rate of the reaction of the thiol group with DTNB was monitored by following the release of thionitrobenzoate anions in the spectrophotometer at 412 nm with respect to time. The molar extinction coefficients of the thionitrobenzoate anion, ε_{412}, in native solvents and 6 M GdnHCl were 13600 $M^{-1}cm^{-1}$ (Ellman, 1959) and 13880 $M^{-1}cm^{-1}$

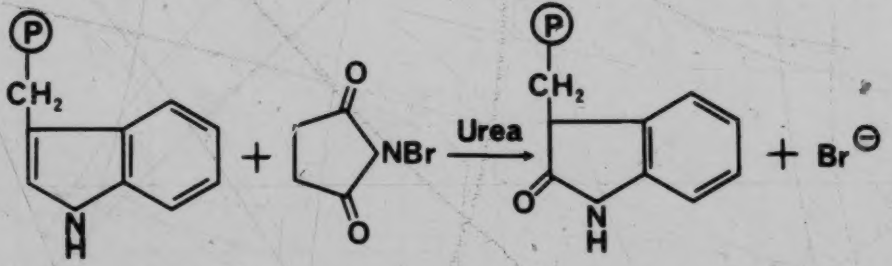
(Gething and Davidson, 1972), respectively. In 8 M urea, an ε_{412} value of 14290 $M^{-1}cm^{-1}$ was used (Gething and Davidson, 1972).

2. Nitration of the α-subunit with tetranitromethane. Nitration of the tyrosyl residues in the α-subunit was achieved by treatment with tetranitromethane, TNM (Sigma Chemicals). The reaction takes the form:



Prior to the nitration reaction, the α-subunit was dialyzed overnight versus 2 changes of 50 mM Tris/HCl, 1 mM EDTA, 1 mM DTT, pH 8.0, with the reducing agent omitted from the second change of dialysis buffer. Nitration of the α-subunit was carried out by the addition of an aliquot of a stock solution of TNM (0.84 M solution in 95% ethanol) to the protein (6.3×10^{-5} M) in 50 mM Tris/HCl, pH 8.0, 1 mM EDTA. The ratio of reagent to protein was 100:1 (or 50:1 Tyr). After reacting for 4 hrs at room temperature, the modified α-subunit was separated from the trinitromethane anion side product by desalting on a Biorad P6DG column equilibrated with 50 mM NH_4HCO_3, 1 mM EDTA, pH 8.0. The product was lyophilized from the pooled fractions. Further purification of the nitrated α-subunit was achieved using reversed-phase HPLC on a Synchropak C-18 column (250 mm x 10 mm I.D.). The lyophilized protein was dissolved in 0.1% TFA and chromatographed using the following conditions: Solvent A consisted of 0.1% TFA, 10% acetonitrile and solvent B was 0.1% TFA, 60% acetonitrile at 1% B/min gradient and a flow rate of 3 ml/min at 25°C. The concentration of the nitrotyrosine was estimated from the absorption at 381 nm using 2200 $M^{-1}cm^{-1}$ as the molar extinction coefficient (Sokolovsky *et al.*, 1966). The concentration of the nitrated protein was determined by amino acid analysis as described earlier in Section IIB. When calculating the mean ellipticity values of the CD spectra of the nitrated protein, a mean residue molecular weight of 111.374 was used.

3. Oxidation of the α-subunit with N-bromosuccinimide. The reaction of tryptophan residues with NBS takes the form:



Prior to oxidation of the α-subunit with NBS (Sigma Chemicals) the cysteine at position 85 of S-100α was protected with the thiol specific reagent, DTNB, as described previously. The reaction with DTNB was performed with a sample of S-100α (1.3×10^{-4} M) that had been passed through a Biorad P6DG column equilibrated in 8 M urea, 0.1 M Tris/HCl, pH 8.0, to remove traces of 2-mercaptoethanol. After reacting with a 9-fold excess of DTNB at room temperature, excess reagent was removed by passage through a Biorad P6DG column equilibrated with 8 M urea, 0.1 M MES, pH 5.5, 0.5 mM EDTA. Oxidation of the DTNB modified α-subunit was carried out at room temperature by the addition of aliquots of stock NBS (20 mM dissolved in 20 mM Mops, pH 7.0) to 1.9 mls of protein solution (approx. 1.1×10^{-4} M) in 8 M urea, 0.1 M MES, pH 5.5, to give a final reagent to protein ratio of approximately 10:1. The reaction was monitored by following the decrease in A_{280} in a spectrophotometer and the final decrease in absorbance was recorded. The number of modified tryptophan residues was estimated by using the following equation as described by Spande and Witkop, (1967b):

$$\%\ \text{tryptophan oxidized} = \frac{\Delta A_{280}}{A_{280}} \cdot 100 \cdot \frac{1.31}{\text{MW}/1000} \cdot \frac{V}{5.5 f n V} \qquad [8]$$

where:

A_{280} = initial absorbance at 280 nm,

ΔA_{280} = decrease in absorption that has been corrected for dilution,

MW = molecular weight (=10,400),

V = initial volume

f = extinction coefficient converting A_{280} of protein into concentration in mg/ml (ε_{280} = 0.893, Baudier *et al.*, 1986),

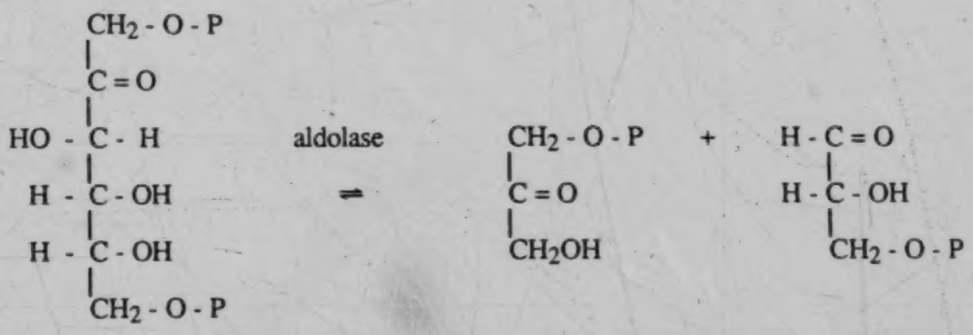
n = number of tryptophans, and

1.3 = empirical factor.

After oxidation of the α-subunit with NBS, the modified protein was dialyzed for 18 hrs against 2 changes of 8 M urea, 0.1 M MES, 0.5 mM EDTA, pH 5.5, to remove excess reagent. The regeneration of the protected sulfhydryl in the NBS oxidized α-subunit as well as a control sample that had not been treated with NBS was achieved by dialysis versus 2 changes of 8 M urea, 0.1 M Tris/HCl, pH 8.0, 2 mM EDTA, 15 mM 2-mercaptoethanol. In order to prepare for the spectroscopic studies, the samples including the NBS oxidized α-subunit, the control sample with the reactivated sulfhydryl, as well as a sample of "native" S-100α that had been subjected to identical treatment apart from the omission of DTNB and NBS, were all dialyzed versus 5 changes of chelex treated buffer containing 100 mM Tris/HCl, pH 7.5 and 1 mM DTT. Amino acid analysis was performed to determine the amino acid composition as well as the concentration of the NBS modified protein used in the spectroscopic studies. When calculating the mean ellipticity values of the CD spectra of the oxidized α-subunit, a mean residue molecular weight of 110.929 was used.

J. Enzymatic Assay for Aldolase

Aldolase (EC 4.1.2.13) catalyzes the cleavage of the following reaction:



Fructose-1,6-bisphosphate

Dihydroxyacetone phosphate

Glyceraldehyde-3-phosphate

The aldolase activity was determined using a quantitative colorimetric assay kit obtained from Sigma Diagnostics, based on a modification of the technique described by Sibley and Lehninger (1949). The latter method used hydrazine to trap the product of the enzymatic reaction thus preventing the interconversion of the triose phosphates by triose phosphate isomerase as well as the reformation of fructose bisphosphate. In the Sigma procedure (procedure #750), the reaction product is hydrolyzed at room temperature to the free triose, which under alkaline conditions is converted to hydroxypyruvic aldehyde. The latter reacts with 2,4-dinitrophenylhydrazine to form the corresponding hydrazone. After further addition of alkali, the hydrazone forms an intense, stable purple color (absorbance maximum at 560 nm) that is proportional to activity.

The enzymatic assays were performed according to the instructions described in Sigma procedure 750. The reaction mixture contained 0.48 ml Trizma fluoride buffer solution (0.3 mol/*l*), pH 7.0, 0.77 ml water, 0.06 ml substrate solution (fructose-1,6-bisphosphate) at a stock concentration of 0.123 M and the reaction initiated after the addition of 0.04 ml of diluted enzymic solution. After reacting at 25°C for 5 min, the reaction was stopped by the addition of 0.45 ml of 1.2 N NaOH and then allowed to stand in a 25°C water bath for 20 min. This was followed by the addition of 0.6 ml of aldolase color reagent containing 2,4-dinitrophenylhydrazine and incubated at 37°C for another 30 min. After the final addition of 2.4 ml of 1.2 N NaOH and equilibration at room temperature for 5 min, the absorbance at 560 nm was recorded. The enzymatic activity of the sample was determined by calibrating the A_{560} reading against a standard curve using a calibration solution containing dihydroxyacetone (from Sigma). The aldolase activity obtained under these conditions is reported as Sigma units of enzymatic activity. One Sigma unit of aldolase is defined as the amount of enzyme that will convert 1 nanomole of fructose-1,6-bisphosphate per minute at 37°C under the conditions described in the procedure. Specific activity is defined as the Sigma unit of aldolase activity per milligram of protein. For the crude enzyme preparation obtained during the fractionation procedure, the concentration of the protein was established using the Bradford assay (Bradford, 1976) with bovine serum albumin as a standard.

In the enzymatic assays employed to test the effect of S-100 protein on the *in vitro* activity of aldolase C, the enzyme purified from bovine brain was used. The protein con-concentration of the enzyme was determined spectrophotometrically using an $E_{1cm,280}^{1\%}$ value of 8.8 for aldolase C (Penhoet *et al.*, 1969b). In addition, a molecular weight of 160,000 (Kawahara and Tanford, 1966) was used when calculating the concentration of the aldolase protomer in moles/liter. In these assays, aliquots of S-100a protein that had been previously dialyzed against 0.1 M Tris/HCl, 1 mM DTT, pH 7.5, were added to the assay reagent containing 1 mM EGTA or Ca^{2+}, prior to starting the reaction by adding 10 µl of diluted enzyme solution (at a concentration of 0.035 mg/ml or 0.21 µM). The aldolase activity observed in the absence of S-100a protein (i.e., basal activity) was subtracted from that observed in the presence of S-100a protein (with or without Ca^{2+} present) and expressed as a percentage stimulation of the enzymatic activity induced by S-100.

III. ISOLATION OF THE α-SUBUNIT OF S-100a PROTEIN FROM BOVINE BRAIN[1]

A. Purification of S-100a and S-100b Protein

Bovine brains were used as the starting material for the purification of S-100 protein, the methodology of which is outlined in Fig. 4. More specifically, bovine brains (700-800g), obtained from a local slaughterhouse were homogenized in 100 mM Tris/HCl,pH 8.0, 5 mM 2-mercaptoethanol and 2 mM EDTA, to solubilize Ca^{2+} binding proteins. The homogenate was then centrifuged to remove insoluble material. The supernatant was brought to 35% saturation with respect to ammonium sulfate concentration at pH 8.5 in order to precipitate cyclic nucleotide phosphodiesterase. Following clarification by centrifugation, the pH of the supernatant was adjusted to 4.2 using glacial acetic acid and the ammonium sulfate concentration brought to 95%. It was left to stir overnight during which time most of the S-100 protein was precipitated. The precipitated material was then collected by centrifugation and the pellet resuspended in 400 ml of 100 mM Tris/HCl, pH 8.0, 1 mM free Ca^{2+}, 1 mM Mg^{2+}. After heat treatment in a boiling water bath for 2-3 min, followed by rapid cooling on ice, it was centrifuged. Whilst the pellet contained the bulk of denatured extraneous protein, the supernatant containing most of the heat stable Ca^{2+} binding proteins, was dialyzed against the starting buffer of the first column, i.e., anion exchange chromatography on DE-52 cellulose. From the elution profile in Fig. 5A, a large proportion of heat stable contaminating protein was removed by washing the column with 50 mM NaCl, followed by 100 mM NaCl. Upon applying a linear gradient of 0.1 to 0.35 M NaCl, several peaks were resolved. From SDS polyacrylamide gel electrophoresis (Laemmli, 1970) (Fig. 5B), it can be seen that peaks I, II and III (lanes c, d and e, respectively) contain contaminating proteins with molecular weights in the range of 29 40 kDa. The S-100 protein which migrated with a subunit M_r of ~ 10,000,

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[1] A portion of the results reported in this chapter has been published: Leung, I.K.M., Mani, R.S. and Kay, C.M. (1986) *Biochem. J.* **237**, 757 - 764.

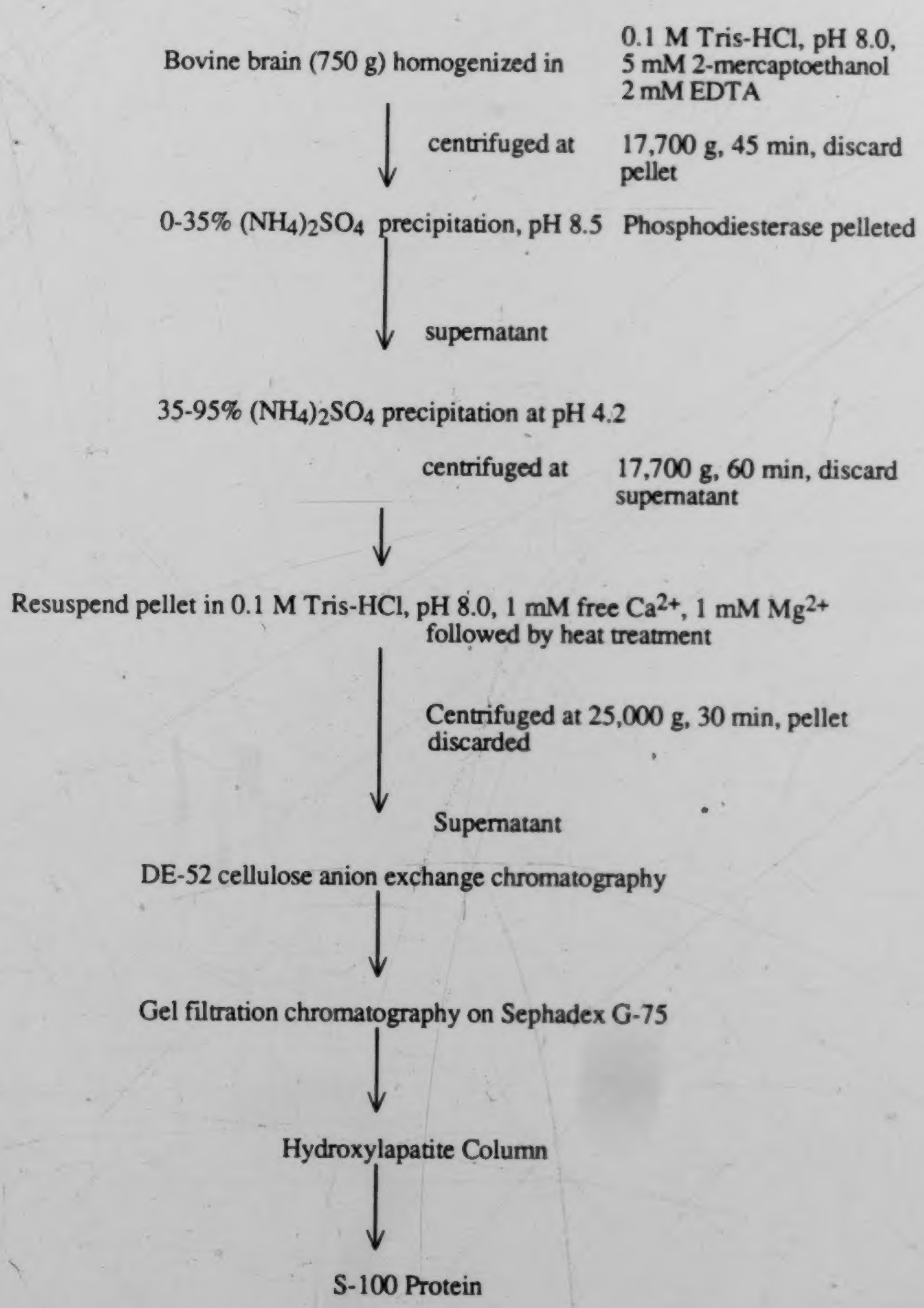
Bovine brain (750 g) homogenized in
0.1 M Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol 2 mM EDTA
centrifuged at
17,700 g, 45 min, discard pellet
0-35% (NH4)2SO4 precipitation, pH 8.5
Phosphodiesterase pelleted
supernatant
35-95% (NH4)2SO4 precipitation at pH 4.2
centrifuged at
17,700 g, 60 min, discard supernatant
Resuspend pellet in 0.1 M Tris-HCl, pH 8.0, 1 mM free Ca2+, 1 mM Mg2+ followed by heat treatment
Centrifuged at 25,000 g, 30 min, pellet discarded
Supernatant
DE-52 cellulose anion exchange chromatography
Gel filtration chromatography on Sephadex G-75
Hydroxylapatite Column
S-100 Protein

Fig. 4 Purification scheme for S-100 protein from bovine brain.

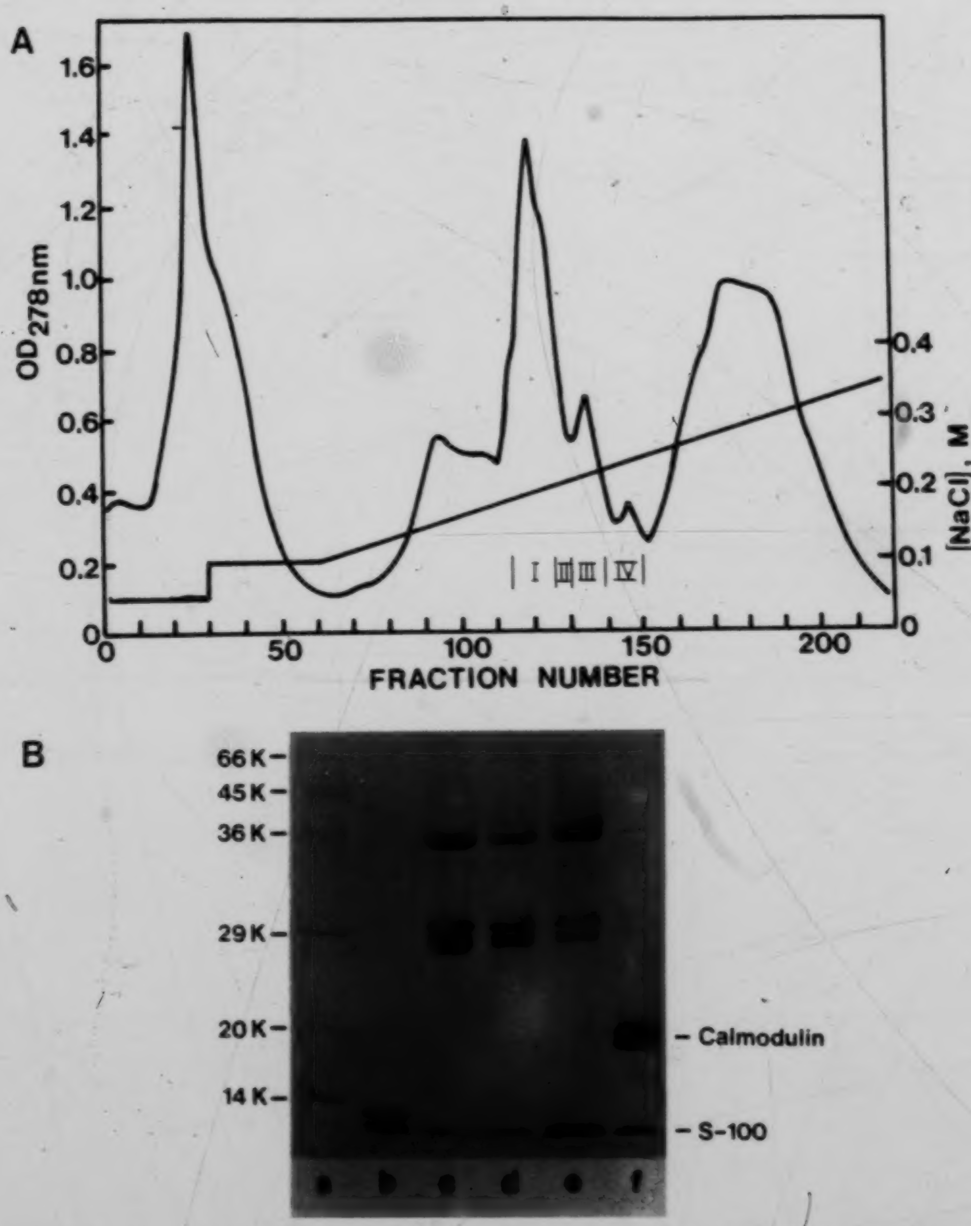

Fig. 5 DE-52 cellulose chromatographic purification of S-100 protein. (A) Chromatographic profile. The solvent was 50 mM Tris/HCl, 50 mM NaCl, 5 mM 2-mercaptoethanol, 2 mM EDTA, pH 7.5. The supernatant obtained after centrifugation of the heat treated protein was dialysed against the starting buffer and applied onto the column (43 x 3 cm), at a flow rate of 40 ml/hr. After washing the column with buffer containing 50 mM NaCl and 100 mM NaCl respectively, the S-100 protein was eluted using a 2 L linear NaCl gradient (0.1 - 0.35 M). (B) SDS polyacrylamide gel electrophoresis (15% gel) of individual fractions. The lanes represent: (a) molecular weight standards; (b) the upper band corresponded to non-proteolysed pike parvalbumin with a M_r of 11,435; (c) fraction 120; (d) fraction 128; (e) fraction 135; (f) fraction 146.

was eluted mainly in peak III (as represented by fraction 135 in lane e). In addition to a small amount of S-100 protein, peak IV (lane f) also contained calmodulin, another Ca^{2+} binding protein that is also abundant in the brain. The material in peak III was pooled, dialyzed and lyophilized. Separation of the S-100 protein from the high molecular weight contaminating material was achieved by using 2 Sephadex G-75 columns arranged in tandem (Fig. 6). SDS PAGE (Fig. 6 inset, lane b) revealed that after the gel filtration step, the S-100 protein (a mixture of S-100a and S-100b) was essentially free of contaminants. Since S-100a (αβ) was used as the starting material for the purification of the α-subunit, most of the S-100b (ββ) was removed from the S-100 mixture using a hydroxylapatite column. In Fig. 7A, it can be seen that upon the application of a step gradient consisting of 0.25 - 0.4 M and 0.4 - 0.85 M ammonium sulfate, S-100 protein was eluted in 3 peaks. The pooled fractions were subjected to further analysis by polyacrylamide gel electrophoresis under native conditions (Davies, 1964). In Fig. 7B, it can be seen that peak I (lane b) and peak IIa (lane c) contained essentially S-100a (>90%), which corresponds to the slower migrating band in a mixture of S-100a and S-100b in lane (a). On the other hand, peak IIb (lane d) and III (lane e) contained both isoforms in approximately equal molar ratios. The protein fractions in peak I and IIa were then dialyzed and lyophilized in preparation for the final column.

B. Isolation of the α-Subunit of S-100a Protein.

At neutral pH, based on the amino acid content of the S-100 protein, the α-subunit possesses a net charge of -12 whereas the β-subunit has a net charge of -14. Hence, anion exchange chromatography was selected as the method of choice for the separation of the α from the β subunit of S-100a protein. Moreover, previous studies indicated the S-100 protein to exist as dimers in native solvents which dissociated into monomers with molecular weight of approximately 10,000 under denaturing conditions (Mani *et al.*, 1982). For this reason, a mixture of S-100 protein that contained predominantly the S-100a isoform, after overnight dialysis versus solvent containing 8 M urea, 50 mM Tris/HCl, 15 mM 2-mercaptoethanol,

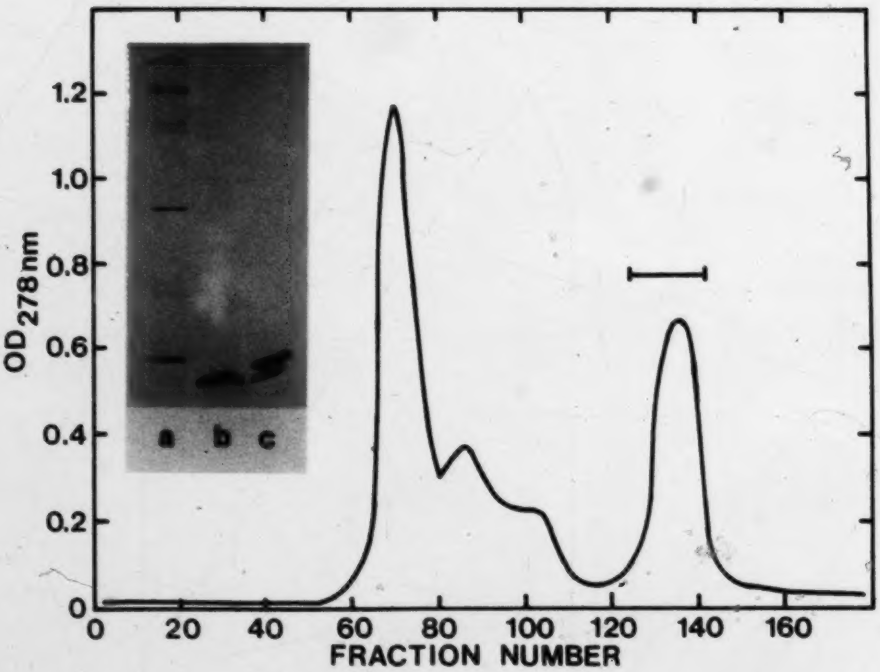

Fig. 6 Purification of S-100 protein by gel filtration chromatography. The lyophilized material pooled from the DE-52 cellulose column was redissolved in 0.1 M Tris/HCl, 0.4 M NaCl, 2 mM EDTA, 5 mM 2-mercaptoethanol, pH 8.0, applied on to two Sephadex G-75 (coarse grade) columns arranged in tandem (90 x 2.5 cm; 115 x 2.5 cm) and equilibrated in the same buffer. A flow rate of 22 ml/hr was used. The inset shows a SDS - PAGE gel (15%) of the pooled fractions of S-100 protein (I——I) in lane (b), with parvalbumin as a molecular weight standard in lane (c) and, in lane (a), molecular weight standards identical to those shown in Fig. 5B.

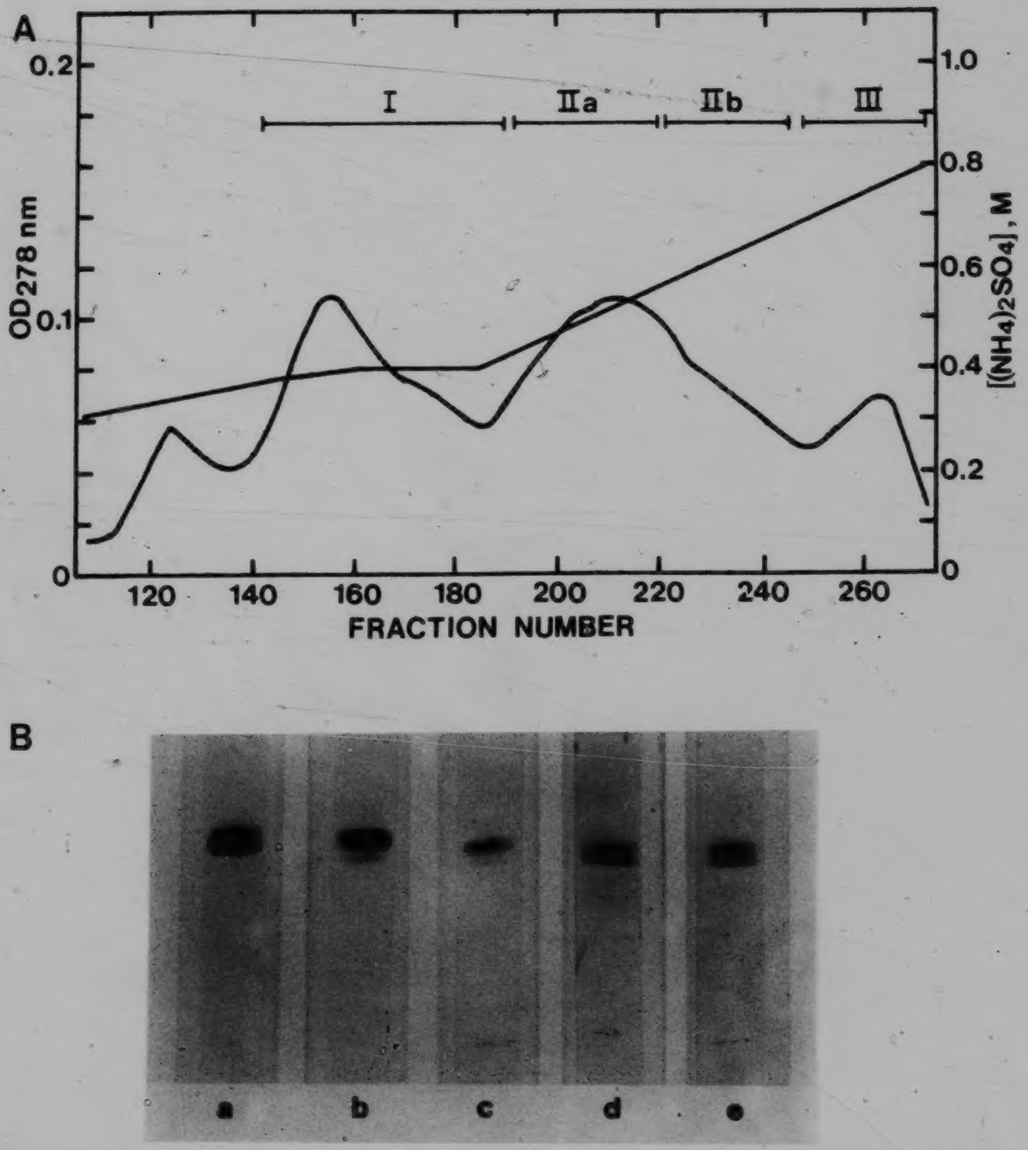

Fig. 7 Hydroxylapatite chromatographic purification of S-100 protein. (A) Chromatographic profile. The lyophilized material recovered from Sephadex G-75 column containing a mixture of S-100a and S-100b was redissolved in 0.1 M Tris/HCl, 0.1 M $(NH_4)_2SO_4$, 1 mM imidazole, 0.2 M NaCl, pH 7.3, and applied on-to the column (30 x 2.2 cm) at a flow rate of 30 ml/hr. After washing the column with buffer containing 0.1 M and 0.25 M $(NH_4)_2SO_4$, respectively, S-100a and S-100b proteins were eluted using a step gradient of 400 ml each of 0.25 - 0.4 M and 0.4 - 0.85 M $(NH_4)_2SO_4$. (|——|) represents fractions pooled, of which peak I and IIa, after dialysis and lyophilization, were subsequently applied on to an anion exchange column. (B) Non-denaturing PAGE of pooled fractions. The lanes represent: (a) a mixture of S-100a and S-100b protein; (b) Peak I; (c) peak IIa; (d) peak IIb; (e) peak III of the hydroxylapatite column.

0.1 M NaCl, 2 mM EDTA, pH 8.0, was applied onto a DEAE Sephadex A-25 anion exchange column equilibrated with the same buffer. Under these conditions, the α-subunit was eluted in the ascending portion of peak I (Fig. 8); peaks II and III also contained α-subunit but with increasing amounts of β-subunit as a contaminant. The material in peak IV was essentially the β-subunit. The amount of protein recovered in peak I as α-chain corresponded to roughly 10-15% of the S-100 protein applied on to the column and was used in the physico-chemical studies reported in the latter chapters. The final yield of α-subunit obtained from 3 kg of bovine brain was approximately 30 mg.

The protein recovered from the anion exchange column was tested for homogeneity by amino acid analysis. A comparison of the amino acid composition of the purified α-subunit with that of Isobe and Okuyama (1981a), as summarized in Table I, revealed excellent agreement between the 2 preparations. The purified α-subunit lacked the single arginine which is characteristic of the β-subunit and contained the single tryptophan found in S-100 protein. The homogeneity of the purified α-subunit was also examined by disc polyacrylamide gel electrophoresis in the presence as well as in the absence of SDS (Fig. 9). In SDS-PAGE (lane a), the α-subunit was essentially homogeneous with a Mr of 10,000 ± 500 as deduced from its mobility in a 12.5% gel, with reference to the mobilities of several protein standards which included: parvalbumin, Mr 11,435 (Gerday, 1976); calmodulin, Mr 16,500 (Klee, 1977); cardiac troponin-C, Mr 18,000 (Van Eerd and Takahashi, 1975); cardiac troponin-I, Mr 27,000 (Hincke, 1981); cardiac troponin-T, Mr 36,000 (Hincke, 1981). Isobe *et al.* (1981a), using SDS-PAGE in 6 M urea, demonstrated that the α-subunit of S-100a protein could be separated from the β-subunit. Hence, under similar conditions, the purified α-subunit in lane b, was observed to be free from any contamination by the β-subunit, which corresponded to the faster migrating band in S-100a (lane c). Polyacrylamide gel electrophoresis was also performed under native conditions (lanes d, e). After alignment of the bromophenol blue dye front, it can be seen that the α-subunit moved faster in the presence of EDTA (lane d). The decrease in mobility in the presence of Ca^{2+} (lane e) could be due to the neutralization of some

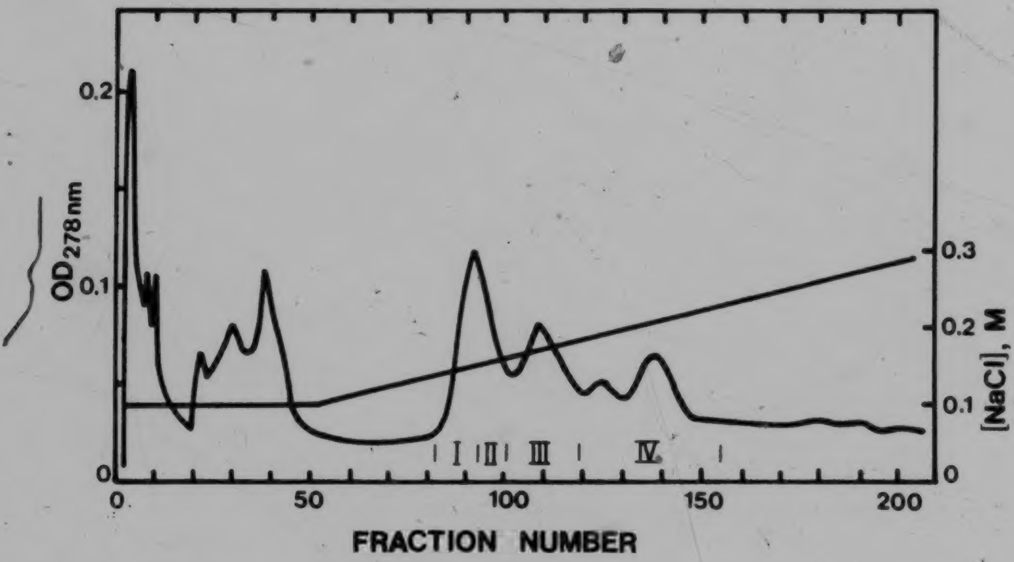

Fig. 8 Chromatographic separation of the α-subunit of S-100a protein on a DEAE Sephadex A-25 column under denaturing conditions. The lyophilized protein pooled from peak I of the hydroxylapatite column was dissolved and dialysed overnight against 8 M urea, 50 mM Tris/HCl, 0.1 M NaCl, 15 mM 2-mercaptoethanol, 2 mM EDTA, pH 8.0, and applied on to the column (2.2 x 16 cm) equilibrated with the same buffer. The α-subunit was eluted with a 900 ml linear NaCl gradient of 0.1 - 0.31 M NaCl, at a flow rate of 22 ml/hr. The fractions in peak I were pooled and used for the characterization of the physico-chemical properties of the α-subunit. Copyright (1986) The Biochemical Society.

TABLE I

Amino acid composition of the α-subunit isolated from bovine brain S-100a protein based on a subunit Mr of 10,400.

	Composition (residues/molecule of protein)		
Amino Acid	α-Subunit	α-Subunit*	β-Subunit*
Lys	8.7	9	8
His	2.0	2	5
Arg	0	0	1
Asx	12.6	13	9
Thr	4.0	4	3
Ser	5.3	5	5
Glx	15.6	15	19
Pro	0	0	0
Gly	6.6	6	4
Ala	8.0	8	5
Cys†	1.0	1	2
Val	7.2	8	7
Met	2.0	2	3
Ile	1.3	1	4
Leu	10.9	11	8
Tyr	2.1	2	1
Phe	4.8	5	7
Trp‡	1.2	1 (93)	0 (91)

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* From the amino acid sequence data of Isobe & Okuyama (1981a).

† Determined by performic acid oxidation.

‡ Determined spectrophotometrically.

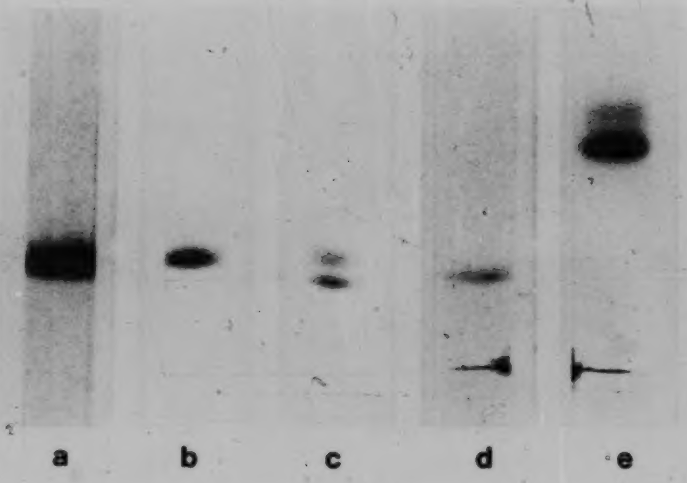

Fig. 9 Electrophoresis of the purified α-subunit of S-100a protein from bovine brain under various conditions. The lanes represent: (a) α-subunit in 12.5% SDS polyacrylamide gel; (b) α-subunit in 6 M urea/SDS/polyacrylamide gel; (c) S-100a protein (αβ) in 6 M urea/SDS/polyacrylamide gel; (d) and (e), the α-subunit in 12.5% polyacrylamide gel in Tris/glycine buffer, pH 8.6, in the presence of 1 mM EDTA (d), and 1 mM Ca^{2+} (e), respectively. Copyright (1986) The Biochemical Society.

of the negative charges resulting from cation binding which has also been observed in calmodulin (Grand *et al.*, 1979). Moreover, in the presence of Ca^{2+}, a second slower migrating diffusely stained band was also observed. Rusca and Calissano (1970) also reported a similar finding and suggested that the state of the sulfhydryl groups in the S-100 protein contributed to the protein electrophoretic heterogeneity under non-denaturing conditions.

C. UV Absorption Spectrum of the α-Subunit

The UV absorption spectrum of the α-subunit of S-100a protein (Fig. 10a) is characteristic of a protein with a high content of aromatic amino acids. Such a property is typical of calcium binding proteins such as calmodulin (Watterson *et al.*, 1980); rabbit skeletal troponin-C (Collins *et al.*, 1977); 21 kDa CaBP (McDonald and Walsh, 1985); β-subunit of calcineurin (Aitken *et al.*, 1984) and pike parvalbumin (Gerday, 1976). The UV absorption spectrum of the α-subunit showed a peak at 278 nm with a shoulder at 283 nm, both of which are contributed by tyrosine chromophores. The appearance of a shoulder around 290 nm characterizes the presence of a tryptophan residue, which distinguishes the α-subunit from the β-subunit. This is also apparent in the derivative spectrum, in the form of a negative trough at the same wavelength. The fine structures induced by phenylalanines around the 260 nm region are less prominent in the α-subunit compared with those in S-100a as shown in Fig. 10b (Mani and Kay, 1983a), since the phenylalanine/ tyrosine ratio is only 5:2 for the α-chain as opposed to 4:1 for the S-100a protein. The differences in phe/tyr ratio in the various isoforms of S-100 protein give rise to a A_{278}/A_{260} ratio of 1.65:1 for S-100α as compared to 1.5:1 for S-100a (αβ) and 1:1 for S-100b (ββ) as seen in Fig. 10a, 10b and 10c, respectively. This ratio is useful in identification of the S-100 protein as it is eluted from the DE-52 cellulose column as well as the various isoforms eluted from the hydroxylapatite columns.

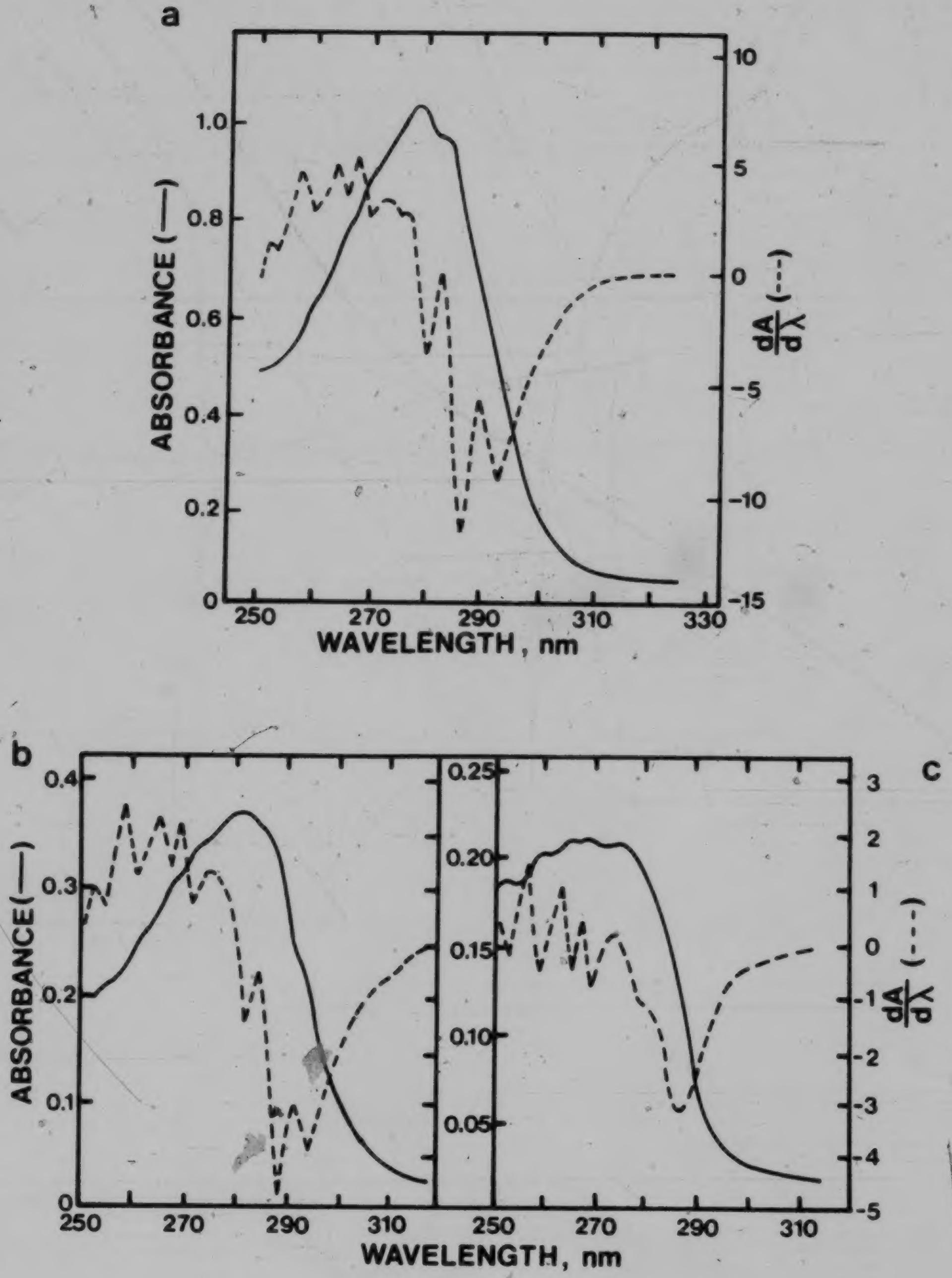

Fig. 10. A comparison of the UV absorption (——) and derivative (------) spectra of (a) the α-subunit of S-100a protein, (b) S-100a protein and, (c) S-100b protein. The proteins were dissolved in 0.1 M Tris/HCl, pH 7.5, 5 mM 2-mercaptoethanol and in the absence of metal ions. Fig. b and c are reprinted with permission from Mani, R. S. and Kay, C.M. (1987) Methods in Enzymology 139, 168. Copyright (1987) Academic Press.

D. Summary

Using anion exchange chromatography in the presence of denaturant, we have separated the α from the β-subunit of S-100a protein. The purified α-subunit is similar in amino acid composition to the α-subunit of S-100a protein sequenced by Isobe *et al.* (1981a). Since the S-100a$_0$ (αα) isoform detected by Isobe *et al.* (1983) also contained an amino acid composition identical to the α-subunit of S-100a protein, it is possible that the α-subunit purified in our laboratory using the procedures described above, corresponds to the same S-100a$_0$ isoform detected by the latter group.

IV. CHARACTERIZATION OF THE PHYSICO-CHEMICAL AND METAL ION BINDING PROPERTIES OF THE α-SUBUNIT OF S-100a PROTEIN[1]

When this study was initiated, it had been demonstrated that both S-100a and S-100b, with a subunit composition of $\alpha\beta$ and $\beta\beta$, respectively, underwent a conformational change upon binding Ca^{2+} and Zn^{2+}. These studies revealed that both Ca^{2+} and Zn^{2+} induced subtle differences in the environment of specific chromophores in the 2 isoforms of the S-100 protein. In order to provide a better understanding of these observed differences, the metal ion binding properties of the α-subunit of S-100a were also examined by a variety of spectroscopic techniques which included CD, UV difference and fluorescence. These studies were performed at pH 7.5 and 8.4, since the S-100 protein is known to exhibit a pH dependence of Ca^{2+} binding. Sedimentation equilibrium studies were also carried out to examine the effects of the metal ions on the weight average molecular weight of S-100α.

A. Binding Stoichiometry of metal ions

1. Ca^{2+} Binding to S-100α. The stoichiometry of Ca^{2+} binding to the α-subunit of S-100a protein was studied using a modification of the equilibrium dialysis technique. Essentially this involved incubating S-100α in the presence of 2-8 fold molar excess of Ca^{2+} and the free Ca^{2+} was then separated from that bound to the protein by ultrafiltration partition across an anisotropic hydrophobic membrane (with a molecular weight cut off of 10,000). A metallochromic indicator, Arsenazo III, was used to determine the amount of free Ca^{2+} present in the ultrafiltrate. The results from these studies, as summarized in Table II, revealed that at pH 7.5, 1 mole of Ca^{2+} was bound per mole of the α-chain. On the other hand, at pH 8.4, 2 Ca^{2+} ions were bound per subunit, suggesting

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[1] A portion of the results [illegible] in this chapter has been published earlier: Leung, I.K.M., Mani,R.S. and Kay, C.M. (1986) *Biochem J.* 237, 757 - 764.

TABLE II

Determination of Number of Ca^{2+} Bound to the α-subunit of S-100a Protein in 100 mM Tris HCl, pH 7.5 and 8.4 containing 15 mM 2-mercaptoethanol

	Moles Ca^{2+} excess/ mole α-subunit	Moles Ca^{2+} bound/* mole α-subunit (mean ± SD)
pH 7.5	2	0.84 ± .03
	3	1.03 ± .08
	4	1.14 ± .09
	8	1.14 ± .15
pH 8.4	3	1.56 ± .04
	4	2.04 ± .09
	6	1.91 ± .13
	8	2.24 ± .15

* The amount of Ca^{2+} bound is expressed as moles bound per mole α-chain monomer in the presence of an excess amount of Ca^{2+} as indicated above. The results represented the mean values obtained from 4 measurements.

The free Ca^{2+} concentration was determined with Arsenazo III, a metallochromic indicator.

The concentration of the protein was 1.64 x 10^{-4} M and 2.24 x 10^{-4} M at pH 7.5 and 8.4, respectively.

that an additional Ca^{2+} binding site became available under more alkaline conditions. These results are in agreement with the binding stoichiometry observed upon monitoring the Ca^{2+} induced conformational change in S-100a and S-100b protein as a function of added Ca^{2+} (Mani *et al.*, 1983; Mani and Kay, 1983a). Attempts were made to study the Ca^{2+} binding stoichiometry at lower molar ratios of Ca^{2+}/subunit; however, they were unsuccessful owing to difficulties in determining the free Ca^{2+} concentration accurately under these conditions.

The stoichiometry of Ca^{2+} binding to the α-subunit was also examined in the presence of 90 mM KCl, since earlier spectroscopic studies suggested that KCl exerted an antagonistic effect towards Ca^{2+} binding to S-100a and S-100b protein (Mani *et al.*, 1983; Mani and Kay, 1983a). As shown in Table III, KCl did not affect the stoichiometry of Ca^{2+} binding to the α-subunit since 2 moles of Ca^{2+} were bound per mole of protein.

2. Zn^{2+} binding to S-100α. The binding stoichiometry of Zn^{2+} to S-100α was also examined using the ultrafiltration partition technique as described for Ca^{2+}. The concentration of the free Zn^{2+} in the ultrafiltrate was determined using the metallochromic indicator, fluorescein. At pH 7.5, when the assay was performed at a protein concentration of 1.73×10^{-5} M, in the presence of 2 - 4.5 moles excess Zn^{2+}/ mole of α-subunit, approximately 0.3 mole of Zn^{2+} was bound per mole of α-chain. Since only a small amount of the Zn^{2+} added was bound to the protein, the assay was repeated using a higher protein concentration of 7.33×10^{-4} M. The result was similar to that obtained previously, suggesting that the α-subunit bound 1 Zn^{2+} ion per chain. Furthermore, the observation that the number of Zn^{2+} ions bound/α-subunit remained low upon increasing the protein concentration to 10^{-4} M, suggested that the protein has a weak affinity for the metal ligand. Since the number of Zn^{2+} bound/subunit leveled off at a protein concentration of 10^{-4} M, the apparent K_d of the α-subunit for Zn^{2+} probably lies in this range. Hence, in the spectral studies reported later, the concentration of Zn^{2+} added to S-100α was approximately

TABLE III

Determination of number of Ca^{2+} Bound to the α-Subunit of S-100a Protein in 100 mM Tris/HCl, 90 mM KCl, 15 mM 2-Mercaptoethanol at pH 8.4

	Moles Ca^{2+} excess/ mole α-subunit	Moles Ca^{2+} bound/* mole α-subunit (mean ± SD)
-KCl	8	2.22 ± .09
	12	1.86 ± .09
+KCl	8	1.77 ± .06
	12	1.77 ± .15

* The amount of Ca^{2+} bound is expressed as moles bound per mole α-chain monomer in the presence of an excess amount of Ca^{2+} indicated above. The results represented the mean values obtained from 3 measurements.

The concentration of the protein was 1.63×10^{-4} M.

10^{-4} M. It was also observed that when the Zn^{2+} concentration exceeded 0.5 mM, the sample became turbid, possibly due to aggregation or precipitation.

B. Spectroscopic Studies on the Effect of Metal Ion Addition to S-100α

1. **Circular Dichroism.** CD in the far UV region (205-250 nm) was used to monitor the conformational change of the α-subunit upon the addition of metal ions. In the absence of metal ions, the spectrum of S-100α in 100 mM Tris/HCl, 15 mM 2-mercapto-ethanol, pH 7.5 (Fig. 11) is characterized by 2 distinctive minima, at 207 and 222 nm, indicative of a protein with high helical content (Manavalan and Johnson, 1983).

Upon the addition of 1 mM Ca^{2+} to the apoprotein, the α-subunit underwent a conformational change, resulting in the $[\theta]_{222}$ value dropping from -16,860 to -15547 ± 300 deg.cm^2.dmol^{-1} (7.8 % decrease). Subsequent addition of 0.2 mM Zn^{2+} to the same sample further decreased the $[\theta]_{222}$ value by another 4% (Table IV). Analysis of the CD data using the method of Chen *et al.* (1974), showed that the α-helical content decreased from 56% to 51% in the presence of Ca^{2+} and this was decreased further by another 1.2% when Zn^{2+} was added to the Ca^{2+} bound protein. When the CD analysis method of Provencher and Glockner (1981) was used, in addition to a 7% decrease in the α-helical content, addition of Ca^{2+} also caused a 21% increase in the β-sheet content of S-100α. On the other hand, upon the addition of Zn^{2+} to the apoprotein, analysis of the CD data (Provencher and Glockner, 1981) suggested that the α-helical and β-sheet content decreased and increased by 8% and 4%, respectively. Moreover, when Ca^{2+} was subsequently added to the protein in the presence of Zn^{2+}, in addition to the 2.7% reduction in the$[\theta]_{222}$ caused by Zn^{2+}, Ca^{2+} was observed to decrease the $[\theta]_{222}$ value by another 10% (Table IV). The CD results suggested that the order of addition of metal ion to the α-subunit was not important, since in both cases, the magnitude of change induced by one metal ion was not sufficiently influenced by the presence of the other, and that the observed final CD spectra were essentially the same. In general, the effect of Ca^{2+} binding

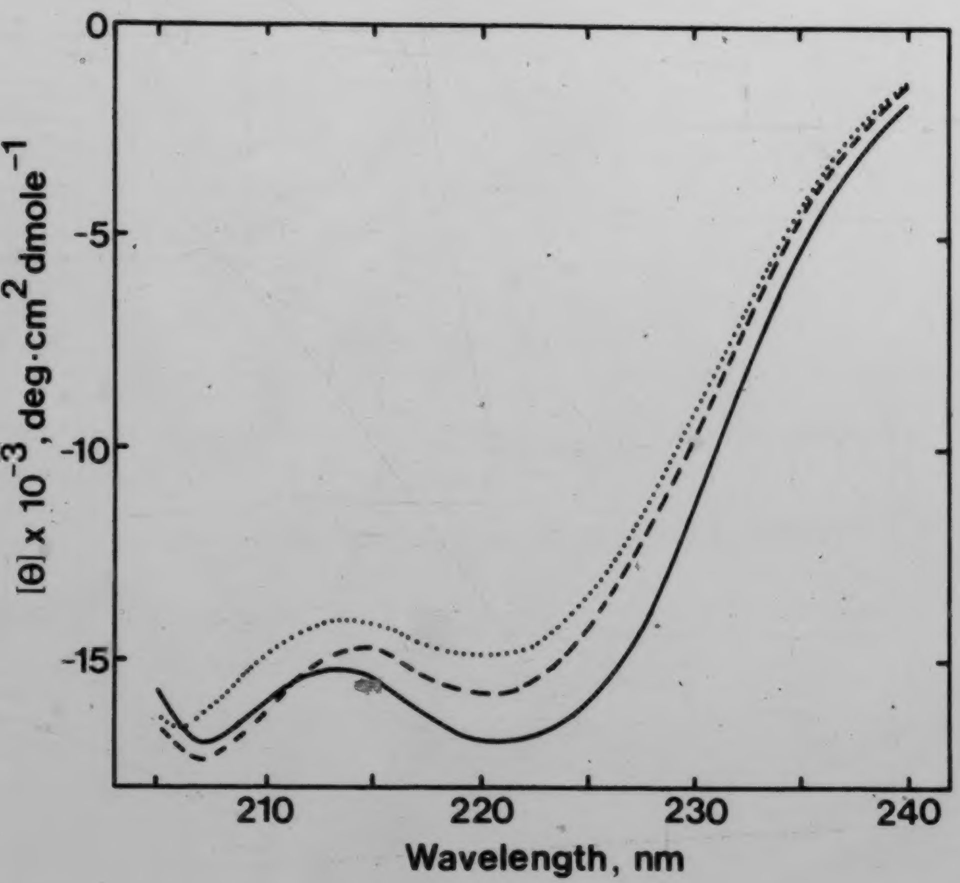

Fig. 11 Far UV CD spectra of the α-subunit of S-100a protein in 0.1 M Tris/HCl, 15 mM 2-mercaptoethanol, pH 7.5, in the absence of Ca^{2+} (——); with 1 mM Ca^{2+} (------), and in the presence of 1 mM Ca^{2+} and 0.2 mM Zn^{2+} (·········). The concentration of the protein was 2[illegible]

TABLE IV

Effect of Ca^{2+} and Zn^{2+} on far UV-CD Parameters upon Binding to the α-subunit of S-100a in 100 mM Tris/HCl, pH 7.5 and 8.4, containing 15 mM 2-mercaptoethanol

		$[\theta]_{222}$	$\Delta[\theta]_{222}$	$\Delta[\theta]_{222}$ %
pH 7.5	α (apo)	-16860	-	-
	α + 1 mM Ca^{2+}	-15547	-1313	7.8
	α + 1 mM Ca^{2+} + 0.2 mM Zn^{2+}	-14846	-2014	11.9
	α (apo)	-16860		
	α + 0.2 mM Zn^{2+}	-16398	-462	2.7
	α + 0.2 mM Zn^{2+} + 1 mM Ca^{2+}	-14632	-2228	13.0
pH 8.4	α (apo)	-16860	-	-
	α + 1 mM Ca^{2+}	-15583	-1277	7.6
	α + 1 mM Ca^{2+} + 0.2 mM Zn^{2+}	-14667	-2193	13.0
	α (apo)	-16860	-	-
	α + 0.2 mM Zn^{2+}	-16410	-450	2.7
	α + 0.2 mM Zn^{2+} + 1 mM Ca^{2+}	-14531	-2329	13.8

$\Delta[\theta]_{222}$ represents the change in the ellipticity value at 222 nm after the addition of various metal ions.

The concentration of the protein was 26 μM and 25 μM, respectively, at pH 7.5 and 8.4.

The standard deviation of the $[\theta]_{222}$ value was ± 300 $deg.cm^2.dmol^{-1}$.

to the α-subunit of S-100a protein resulted in a loss of α-helical content, similar to that observed for S-100a and S-100b (Mani and Kay, 1987a). In this respect, the Ca^{2+} effect on the S-100 protein is different from other calcium binding proteins such as troponin-C and calmodulin where Ca^{2+} binding results in an increase in the apparent helical content of these proteins (Klee, 1977; Dedman *et al.*, 1977; Murray and Kay, 1972). The binding of Ca^{2+} and Zn^{2+} was also examined in 100 mM Tris/HCl, 15 mM 2-mercaptoethanol, pH 8.4. In Table IV, it can be seen that the addition of either Ca^{2+} or Zn^{2+} to the apoprotein at pH 8.4, resulted in a decrease in the $[\theta]_{222}$ values, very similar to those observed at pH 7.5. Moreover, irrespective of the order of addition of the metal ions, the observed final spectra were similar and resembled those recorded at pH 7.5, suggesting that at these pH values, both Ca^{2+} and Zn^{2+} can bind to S-100α and induce a gross conformational change in the presence of each other.

In addition to studying the effect of Ca^{2+} binding at pH 7.5 and 8.4, the effect of this metal ion was also examined at a lower pH range. As seen in Table V, addition of 1.6 mM Ca^{2+} to the apoprotein resulted in a decrease in the $[\theta]_{222}$ values, and this observed decrease was not affected as the pH was varied from 5.6 to 8.4. Nevertheless, Ca^{2+} exerted slightly different effects on the α-subunit at pH 7.5 and 8.4, based on fluorescence and near UV CD results reported later.

The effect of K^+ and Mg^{2+} on the α-subunit is reported in Table VI. Far UV CD studies indicated that the addition of 2 mM Mg^{2+} to S-100α induced only a small conformational change, whereas KCl (~ 90 mM) was without any effect at pH 7.5 and 8.4.

CD measurements in the near UV region (250-320 nm) were also employed to examine the effect of metal ion binding on the tertiary structure of the α-subunit. A representative near UV CD spectrum is shown in Fig. 12. The negative band at 297 nm is due to the single tryptophan residue. The positive bands at 278 and 284 nm are attributed to tyrosine residues while those at 261.5 and 268.5 are assigned to one or more of the phenylalanines of the protein. Addition of 1 mM Ca^{2+} to the apoprotein at pH 7.5 resulted

TABLE V

Far UV-CD Parameters of the α-subunit of S-100a Protein as a Function of pH

pH (buffer system)[a]	$[\theta]_{222}$ ($-Ca^{2+}$)[b]	$[\theta]_{222}$ ($+Ca^{2+}$)[b]	$\Delta[\theta]_{222}$, %
5.60 (MES)	-16232	-14534	10.7
6.05 (MES)	-16421	-15018	8.5
6.50 (PIPES)	-16138	-14533	9.9
6.81 (PIPES)	-16421	-15018	8.5
7.12 (MOPS)	-16327	-14921	8.6
7.51 (MOPS)	-16610	-15100	9.1
8.42 (Tris)	-16421	-14725	10.3

[a] The ionic strength of the buffers was 100 mM.

[b] The standard deviation of the $[\theta]_{222}$ value was ± 300 $deg.cm^2.dmol^{-1}$.

S-100α (22.6 μM) was dissolved in the buffers as described above and in the presence of 1 mM EDTA and 15 mM 2-mercaptoethanol. Far UV CD spectra were recorded at various pH values in the presence of 1 mM EDTA and after the addition of 2.6 mM Ca^{2+} (i.e., 1.6 mM free).

TABLE VI

Effect of K^+ and Mg^{2+} on Far UV-CD Parameters of S-100α in 100 mM Tris/HCl, pH 7.5 and 8.4, Containing 15 mM 2-Mercaptoethanol

		$[\theta]_{222}$	$\Delta[\theta]_{222}$	$\Delta[\theta]_{222}$, %
pH 7.5	α (apo)	-16860		-
	α + 87 mM KCl	-16550	-100	-
	α + 1.96 mM Mg^{2+}	-16360	-500	3.0
pH 8.4	α (apo)	-16860		
	α + 60 mM KCl	-16660	-200	1.1
	α + 1.96 mM Mg^{2+}	-16505	-355	2.1

The standard deviation of the $[\theta]_{222}$ value was ± 300 $deg.cm^2.dmol^{-1}$.

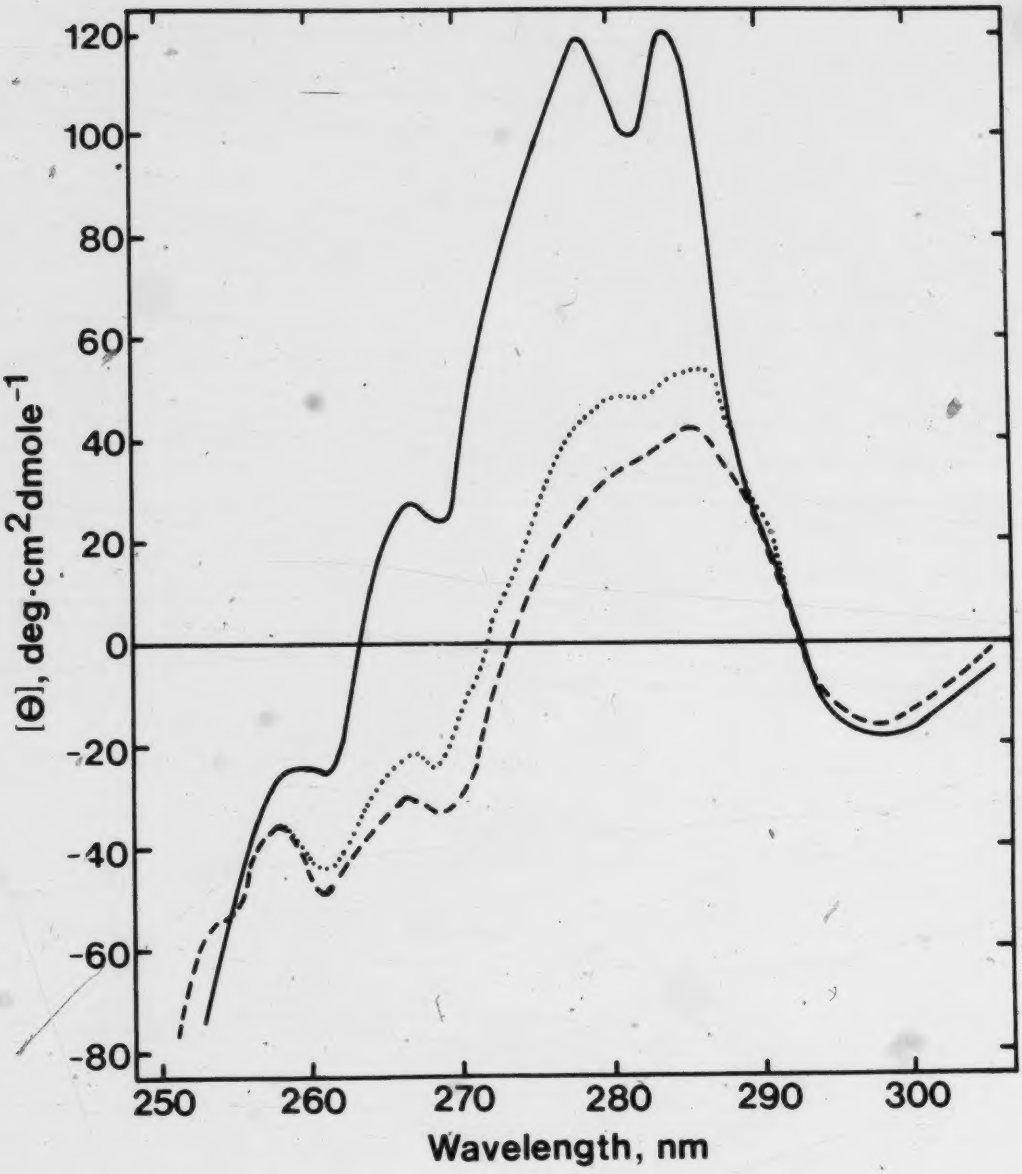

Fig. 12 Near UV CD spectra of the α-subunit of S-100a protein in 0.1 M Tris/HCl, 15 mM 2-mercaptoethanol, pH 7.5, in the absence of Ca^{2+} (——); with 1 mM Ca^{2+} (------), and in the presence of 1 mM Ca^{2+} and 87 mM KCl (········).

in the perturbation of the tryptophan band at 297 nm and the tyrosine bands in the 280 nm region of the spectrum. Ellipticity values at 261.5 and 268.5 nm were also affected. While the addition of KCl has minimal effect on the environment of the aromatic chromophores (Table VII), it can be seen from Fig. 12 that the subsequent addition of K^+ to Ca^{2+} bound protein antagonized the effect induced by Ca^{2+}. Moreover, when the order of addition was reversed, Ca^{2+} could induce a conformational change in the presence of KCl. Zn^{2+}, on the other hand, when added to the metal free α-subunit at a concentration equivalent to the total Zn^{2+} present in the brain (0.4 mM) (Frederickson *et al.*, 1983), resulted in a small alteration in the environment near the tyrosines only (Table VII). Mg^{2+} also has a minimal effect on the tertiary structure of the protein.

The effect of Ca^{2+} binding to the α-subunit at pH 8.4 was similar to that observed at pH 7.5, i.e., perturbation of the environment near the aromatic chromophores (Table VII). However, addition of Ca^{2+} to the α-subunit at pH 8.4 resulted in a 55% reduction in the $[\theta]_{284}$ value compared to a 67% reduction at pH 7.5. Since CD results from a difference in the absorption of left and right circularly polarized light by carbon asymmetry centers in amino acids, the differences observed in the $[\theta]_{284}$ values suggest that Ca^{2+} affects the environment of these asymmetric centers to different extents at pH 7.5 and 8.4. When the effects of other metal ions were compared with that of Ca^{2+}, addition of K^+, Mg^{2+}, or Zn^{2+} to the apoprotein only induced a small effect in the near UV region of the CD spectra (Table VII). However, when K^+ was subsequently added to Ca^{2+} bound protein, K^+ was able to antagonize the effect induced by Ca^{2+}.

CD titration of S-100α with Ca^{2+} was performed by monitoring the decrease in the ellipticity value at 278 nm since the tyrosines were most affected upon the addition of this metal ion. The titration curves, obtained at pH 7.5 and 8.4 (Fig. 13), were both monophasic, suggesting the presence of one class of Ca^{2+} binding site, with apparent K_d values of $3.34 \pm 0.2 \times 10^{-4}$ and $1.26 \pm 0.2 \times 10^{-4}$ M, respectively. Analysis of CD titration data at pH 8.4, using a Scatchard plot which is based on the assumption that the

TABLE VII

Effect of Metal Ions on Ellipticity Values of Tryptophan and Tyrosines upon Addition to α-subunit in 100 mM Tris/HCl, pH 7.5 Containing 15 mM 2-mercaptoethanol

		$[\theta]_{297}$*	$[\theta]_{284}$*	$[\theta]_{278}$*
pH 7.5	α (apo)	-17.8	120.3	119
	α + 1 mM Ca^{2+}	-16.4	39.7	27.4
	α + 1 mM Ca^{2+} + 87 mM KCl	-16.4	52	43.8
	α (apo)	-17.8	121.7	120.3
	α + 87 mM KCl	-20.5	124.4	128.6
	α + 87 mM KCl + 1 mM Ca^{2+}	-13.7	56	49.2
	α (apo)	-17.8	120.3	123
	α + 0.2 mM Zn^{2+}	-17.8	123.1	127.2
	α + 0.4 mM Zn^{2+}	-17.8	127.2	132.7
	α (apo)	-17.8	120.4	117.6
	α + 1.96 mM Mg^{2+}	-19.1	124.5	123.1
pH 8.4	α (apo)	-15.5	117.6	117.3
	α + 1 mM Ca^{2+}	-6.4	52.9	47.7
	α + 1 mM Ca^{2+} + 87 mM KCl	-6.4	64.5	54.1
	α (apo)	-14.2	117.3	118.6
	α + 0.2 mM Zn^{2+}	-14.2	121.2	121.2
	α + 87 mM KCl	-18.0	119.9	121.2
	α + 87 mM KCl + 1 mM Ca^{2+}	-7.7	64.5	63.2
	α + 1.96 mM Mg^{2+}	-14.2	121.3	123.9

* The standard deviation in the ellipticity values in these experiments was ± 2.7 deg.cm^2.dmol^{-1} and ± 2.6 deg.cm^2.dmol^{-1} at pH 7.5 and 8.4, respectively.

The concentrations of the protein were 77.99 μM and 82.73 μM at pH 7.5 and 8.4, respectively.

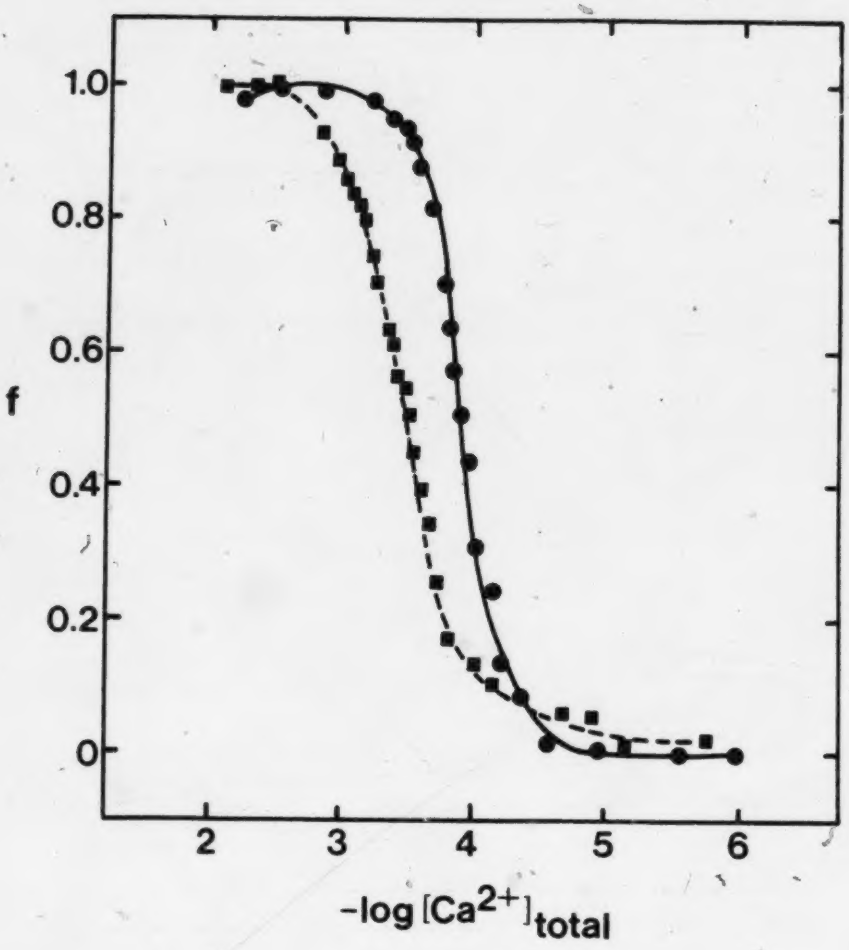

Fig. 13 CD titration of the α-subunit of S-100a protein in 100 mM Tris/HCl, 15 mM 2-mercaptoethanol at pH 7.5 (-------) and pH 8.4 (——), as a function of the concentration of added Ca^{2+}. The titration was monitored by following the changes in the ellipticity value at 278 nm, which is expressed as fractional conformational change. The concentration of the protein was 112.7 μM and 82.7 μM at pH 7.5 and 8.4, respectively.

conformational change was completed upon Ca^{2+} binding to both sites, revealed a pronounced downward curvature (Fig. 14). This suggests that at pH 8.4, the 2 Ca^{2+} binding sites on S-100α interact in a positively cooperative manner and that the K_d value obtained from the titration curve most likely represents the average affinity of the 2 sites.

2. UV Difference Spectroscopy. The effects of metal ions on the microenvironment of the α-subunit were also studied by monitoring the ultraviolet difference spectra produced by these perturbants.

A typical Ca^{2+} induced difference spectrum for the α-subunit at pH 7.5 is shown in Fig. 15. The spectrum is generated by adding a small aliquot of 0.1 M $CaCl_2$ stock solution to the sample cell and an equal volume of chelex treated water to the reference cell. The negative difference peak at 292.5 nm is attributed to the single tryptophan while those at 284.5 and 277.5 nm are due to one or both of the tyrosines in the α-subunit. These minima characterized blue shifts in the absorption bands of the corresponding aromatic residues and are usually interpreted as arising from an increased exposure of these aromatic chromophores to solvent (Donovan, 1973). In addition, a sharpening of the fine structure of the absorption bands below 270 nm is observed, suggesting one or more of the phenylalanines are perturbed in the presence of Ca^{2+}. These findings are in agreement with the near UV CD results whereby the environments of the tryptophan, tyrosine and phenylalanine residues are perturbed upon binding Ca^{2+}. The subsequent addition of 0.1 mM Zn^{2+} to the Ca^{2+} saturated protein resulted in a slight reduction in the $\Delta\varepsilon$ values of the difference peaks assigned to tyrosine (Table VIII), suggesting that the tyrosines moved to a less polar environment. The effect of Ca^{2+} binding to the α-subunit was also studied at pH 8.4. As evident in Table VIII, at pH 8.4,Ca^{2+} perturbed the environment of the tryptophan and the tyrosines. Furthermore, the magnitudes of the difference peaks assigned to these chromophores were on average 7 % larger than the values observed at pH 7.5. However, the fact that the absorbance of aromatic amino acids is pH dependent (Donovan, 1973), suggested that the small discrepancy in the magnitudes of the difference peaks observed in

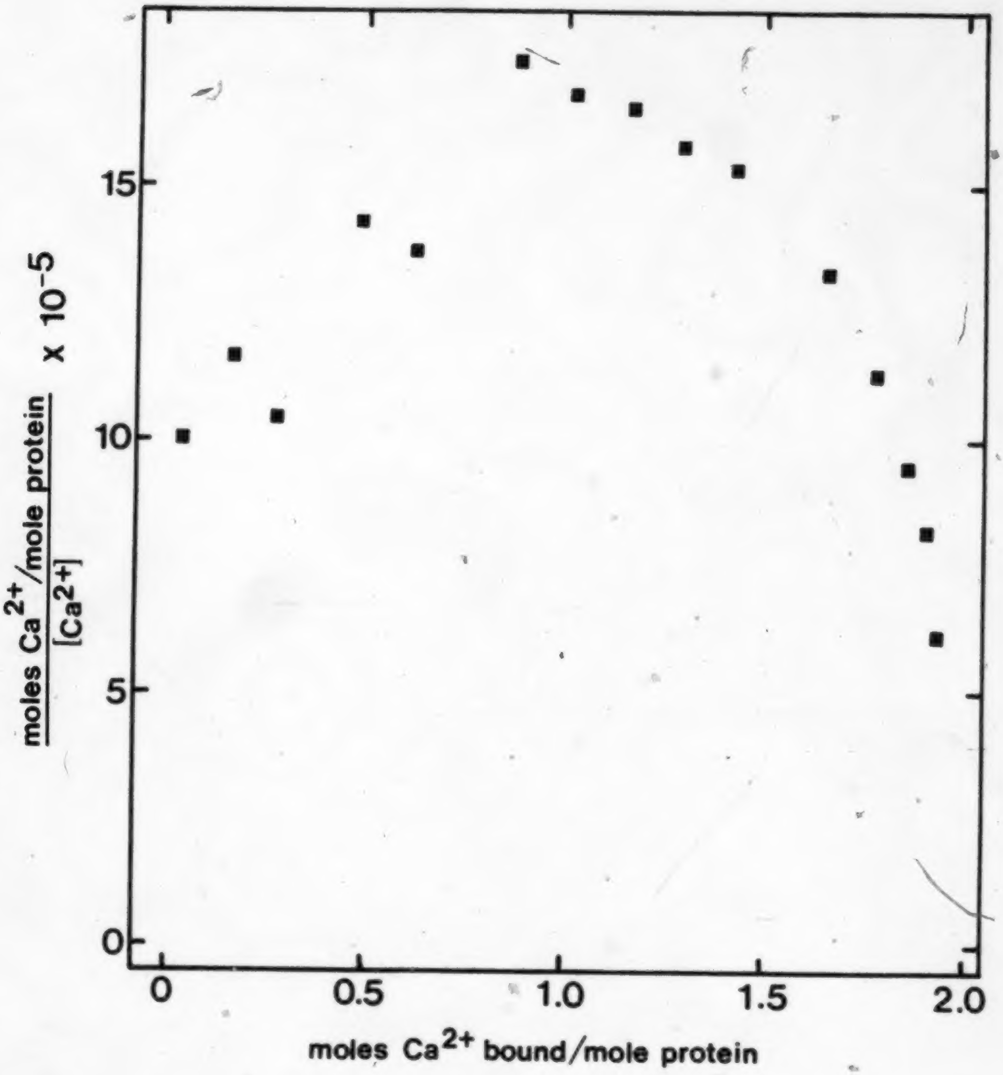

Fig. 14 Scatchard analysis of the CD titration data at pH 8.4. To estimate the fraction of Ca^{2+} bound to the α-subunit, the decrease in the ellipticity value at 278 nm after each addition of Ca^{2+} was expressed as a ratio of the observed final decrease in $[\theta]_{278}$ value upon saturation of the protein with Ca^{2+}. At the end of the titration, the number of the Ca^{2+} bound was assumed to be 2 since binding stoichiometry experiments revealed 2 Ca^{2+} binding sites exist on each α-subunit at pH 8.4. The concentration of the Ca^{2+} bound to the protein was calculated from the product of the protein concentration (82.7 μM) and the fraction of Ca^{2+} bound, which upon subtracting from the total Ca^{2+} added, gave the free Ca^{2+} concentration.

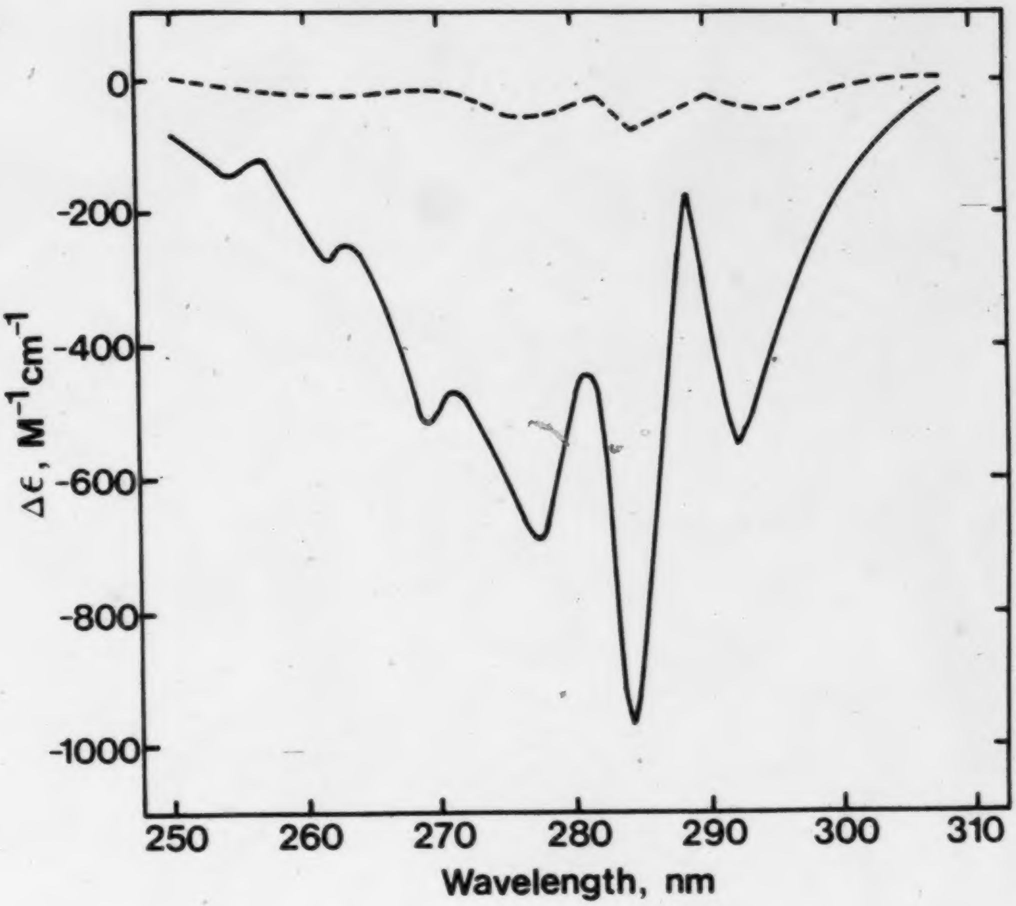

Fig. 15 UV difference spectra of the α-subunit of S-100a protein induced by the addition of either 1.67 mM Ca^{2+} (——) or 0.1 mM Zn^{2+} (------). The solvent system was 0.1 M Tris/HCl, pH 7.5, and the protein concentration was 27 μM.

TABLE VIII

Effect of Addition of Metal Ions to the α-subunit of S-100a Protein in 100 mM Tris/HCl pH 7.5 and 8.4 as Studied by UV Difference Spectroscopy

		$\Delta\varepsilon_{292.5}$	$\Delta\varepsilon_{284.5}$	$\Delta\varepsilon_{277.5}$	$\Delta\varepsilon_{269}$	$\Delta\varepsilon_{261.5}$
pH 7.5	α + 1.67 mM Ca^{2+}	-555	-986	-709	-522	-269
	α + 1.66 mM Ca^{2+} + 0.1 mM Zn^{2+}	-485	-866	-626	-430	-104
	α + 0.1 mM Zn^{2+}	-49	-74	-54	-41	-14
	α + 0.1 mM Zn^{2+} + 1.66 mM Ca^{2+}	-505	-926	-702	-530	-285
	α + 87 mM KCl	-33	0	-33	-60	-55
	α + 87 mM KCl +1.61 mM Ca^{2+}	-484	-935	-713	-525	-304
	α + 1.66 mM Ca^{2+} + 87 mM KCl	-486	-875	-644	-496	-261
	α + 4.96 mM Mg^{2+}	-39	-46	-43	-31	-41
	α + 4.96 mM Mg^{2+} 1.65 mM Ca^{2+}	-595	-1057	-760	-560	-288
pH 8.4	α + 1.67 mM Ca^{2+}	-607	-1046	-749	-537	-286
	α + 1.66 mM Ca^{2+} + 87 mM KCl	-556	-969	-693	-473	-242
	α+ 87 mM KCl	17	41	41	11	0
	α + 87 mM KCl + 1.61 mM Ca^{2+}	-520	-896	-650	-464	-261
	α + 0.1 mM Zn^{2+}	-28	-42	-28	-28	-28

Δε values are reported as $M^{-1}cm^{-1}$ at the corresponding wavelengths indicated above. The standard deviation was ± 13.6 $M^{-1}cm^{-1}$ and 14.1 $M^{-1}cm^{-1}$ at pH 7.5 and 8.4, respectively. The concentration of the protein was 26 μM.

the Ca^{2+} induced spectra at pH 7.5 and 8.4, could be due to a pH induced perturbation of these chromophores.

From the Zn^{2+} induced UV difference spectrum obtained at pH 7.5 (Fig. 15), it can be seen that Zn^{2+} has a small effect. The small change in the $\Delta\varepsilon$ values observed here can be interpreted as a minor conformational change induced by the neutralization of charges on the side chains of amino acids upon metal ion binding to the protein (Nagy *et al.*, 1978). Moreover, the magnitudes of these difference peaks induced by Zn^{2+} at pH 7.5 and pH 8.4, were less than 10% of those observed with Ca^{2+} (Table VIII), implying that the Zn^{2+} effect on the α-subunit is minimal compared to that of Ca^{2+}. The binding of Zn^{2+} to the α-subunit appears not to affect the ability of Ca^{2+} to perturb the microenvironment. This is based on the observation that the difference spectrum generated upon the addition of Ca^{2+} to the Zn^{2+} bound protein, was similar in magnitude to the one obtained by addition of Ca^{2+} alone to the apoprotein (Table VIII).

As evident in Table VIII, K^+ has only a minimal effect on the environment near the phenylalanines. However, when K^+ was subsequently added to the Ca^{2+} saturated proteins, it exerted a small antagonistic effect on the Ca^{2+} induced difference spectrum at both pH 7.5 and 8.4, as indicated by a slight reduction in the $\Delta\varepsilon$ values of the corresponding aromatic residues. Furthermore, Ca^{2+} could bind to S-100α in the presence of KCl, since difference peaks were observed upon the subsequent addition of Ca^{2+} to the protein containing K^+. In the case of Mg^{2+}, addition of this metal ion has a minimal effect on the aromatic chromophores. Furthermore, when the effect due to Mg^{2+} was accounted for, the magnitude of the difference peaks that resulted from the subsequent addition of Ca^{2+} to a protein solution containing Mg^{2+} was similar to those reported for the addition of Ca^{2+} to the apoprotein, suggesting that the presence of Mg^{2+} did not appear to affect Ca^{2+} binding to the α-subunit.

3. Fluorescence Spectroscopy. The fluorescence of nonconjugated protein originates from 3 aromatic amino acids (phenylalanine, tyrosine and tryptophan) which absorb in the near ultraviolet region (Teale and Weber, 1957). In the case of the α-subunit which contains a single tryptophan, its fluorescence will be dominated by this residue due to its strong absorption properties and high quantum yield. Since the tryptophan is highly sensitive to the polarity of its surrounding environment, fluorescence spectroscopy is a useful technique to examine the effect of metal ions on the local environment in the protein when solutions of S-100 are subjected to excitation.

(i) Effect of metal ion on emission spectra

Upon exciting the α-subunit at 280 nm, where both tyrosine and tryptophan absorb, its emission maximum is centered at 339 ± 1 nm (Fig. 16). The emission maximum at 339 nm is due to the partly buried tryptophan (Longworth, 1971) and the shoulder around 307 ± 1 nm is due to the tyrosines. Addition of 1 mM Ca^{2+} at pH 7.5, resulted in a 14.8% increase in the relative fluorescence intensity and the position of the emission maximum shifted to 341 ± 1 nm (i.e., a red shift), suggesting the tryptophan residue became more exposed to solvent after binding Ca^{2+}, in agreement with the difference spectral data. An emission maximum centered at 339 ± 1 nm was also observed upon exciting the apoprotein at 295 nm, i.e., maximally exciting the tryptophan (Table IX). While the addition of Ca^{2+} caused a similar red shift in the emission maximum, the increase in fluorescence intensity was small (4.4%). This suggests that apart from tryptophan, one or both of the tyrosines are also perturbed by Ca^{2+} binding, as evident from the larger fluorescence enhancement observed when both aromatic chromophores were excited at 280 nm. Irrespective of whether Ca^{2+} was present or not, the excitation maximum of the α-subunit was centered at 282 ± 1 nm, upon monitoring the emission at 341 nm. Ca^{2+} binding to the α-subunit was also studied at pH 8.4. Apart from a red shift in the emission maximum, addition of Ca^{2+} at pH 8.4 resulted in a significantly larger increase in the fluorescence intensity than that observed at pH 7.5 (Table IX). The effect of addition of

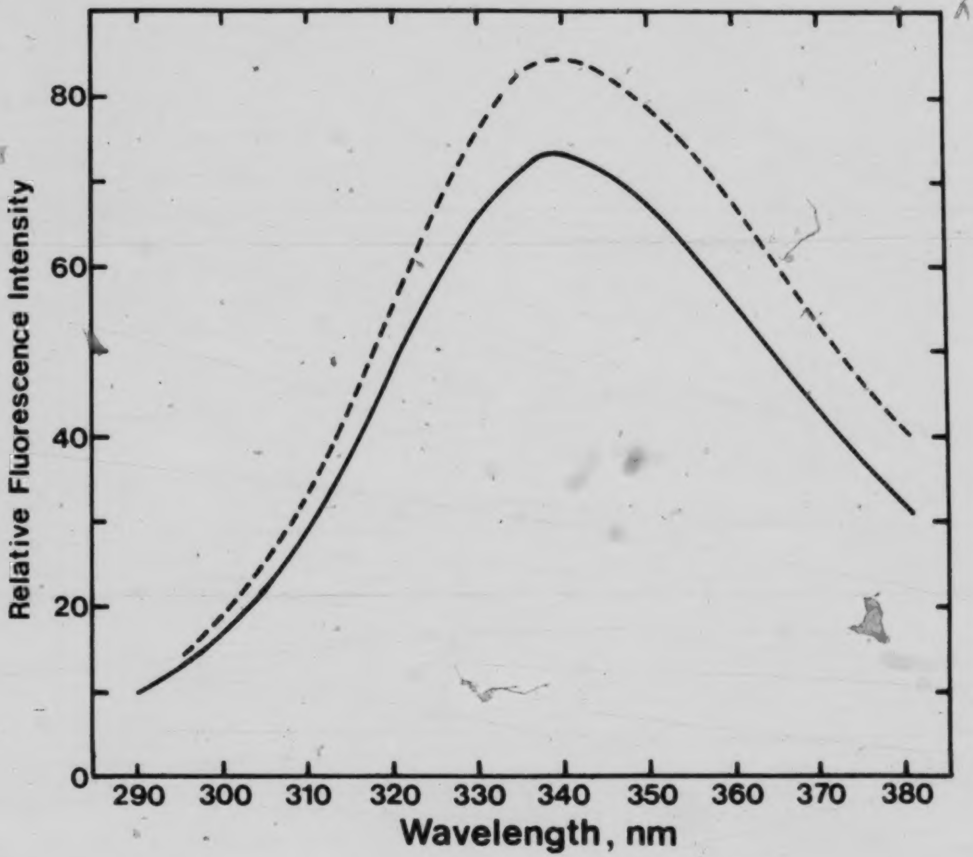

Fig. 16 Fluorescence emission spectra of the α-subunit of S-100a protein in 0.1 M Tris/HCl, pH 7.5, containing 15 mM 2-mercaptoethanol, in the absence of (——), and in the presence of 1 mM Ca^{2+} (-------). The concentration of the protein was 3.38 μM and an excitation wavelength of 280 nm was used.

TABLE IX

Effect of Metal Ion Addition on Emission Spectra of S-100α in 100 mM Tris/HCl, 15 mM 2-mercaptoethanol at pH 7.5 and 8.4

		λ_{ex} *(nm)	$\lambda_{em.max}$ * (nm)	$\Delta F_{em.max}$ (%)
pH 7.5	α	280	339	-
	α + Ca^{2+}	280	341	+14.8
	α	295	339	-
	α + Ca^{2+}	295	341	+4.4
	α + Zn^{2+}	280	339	NC**
	α + K^{+}	280	339	NC**
	α + Mg^{2+}	280	339	NC**
pH 8.4	α	280	339	-
	α + Ca^{2+}	280	341	+28.5
	α	295	339	-
	α + Ca^{2+}	295	341	+16.4
	α + Zn^{2+}	280	339	NC**

λ_{ex} denotes the excitation wavelength employed in the experiment and

$\lambda_{em\ max}$ represents the position of the emission maximum observed from the spectra.

$\Delta F_{em.max}$ represents percentage change in fluorescence intensity observed at the emission maximum before and after the addition of various metal ions.

** denotes no change in fluorescence intensity detected at the emission maximum.

The concentration of Ca^{2+}, Zn^{2+}, K^{+} and Mg^{2+} added were 1 mM, 0.2 mM, 90 mM and 4.98 mM respectively.

The concentration of the protein was 3.38 μM.

either K^+ (90 mM), Mg^{2+} (4.9 mM) or Zn^{2+} (0.2 mM) to the α-subunit was also examined by fluorescence spectroscopy at pH 7.5. Addition of each of the metal ions to the apoprotein has minimal effect on the position of the emission maximum and the fluorescence intensity (Table IX), suggesting that they do not induce any significant change in the environment near the tryptophan residue.

(ii) Fluorescence titrations

Fluorescence titrations were carried out with S-100α in order to estimate the affinity of the protein for Ca^{2+}. By monitoring the percentage change in the fluorescence intensity as a function of the amount of Ca^{2+} added, a monophasic titration curve was obtained (Fig. 17), indicating the existence of one class of Ca^{2+} binding site at pH 7.5, with an apparent K_d value of $1.58 \pm 0.25 \times 10^{-4}$ M at the transition midpoint. Ca^{2+} titration was also carried out in the presence of 90 mM KCl and, the titration curve obtained was identical in shape to that observed in the absence of KCl, with an apparent K_d value of $6.3 \pm 0.25 \times 10^{-4}$ M. The slight decrease in affinity for Ca^{2+} in the presence of KCl observed here, and the results obtained from UV difference spectroscopy, suggested that K^+ only exhibited a small antagonistic effect towards Ca^{2+} binding to the α-subunit. On the other hand, when the titrations were repeated in the presence of either 3 mM Mg^{2+} or 9.9 μM Zn^{2+} (3.55 mole Zn^{2+}/mole dimer), no significant change in the apparent K_d value for Ca^{2+} was observed (see Table X), suggesting that the presence of either of these metal ions did not affect the affinity of the protein for Ca^{2+}.

Fluorescence titration studies of the α-subunit with Ca^{2+} were also performed at pH 8.4. The titration curve, as shown in Fig. 18, is biphasic, revealing 2 classes of Ca^{2+} binding sites with different affinities. While one class of site binds Ca^{2+} with higher affinity (apparent K_d of $5.6 \pm 0.1 \times 10^{-5}$ M) and accounts for approximately 70% of the observed fluorescence enhancement, the other class binds weaker (apparent K_d of $1.88 \pm 0.1 \times 10^{-4}$ M) and only results in a small increase in fluorescence intensity. Inclusion of 90 mM KCl in the protein solution during titration resulted in a monophasic titration curve,

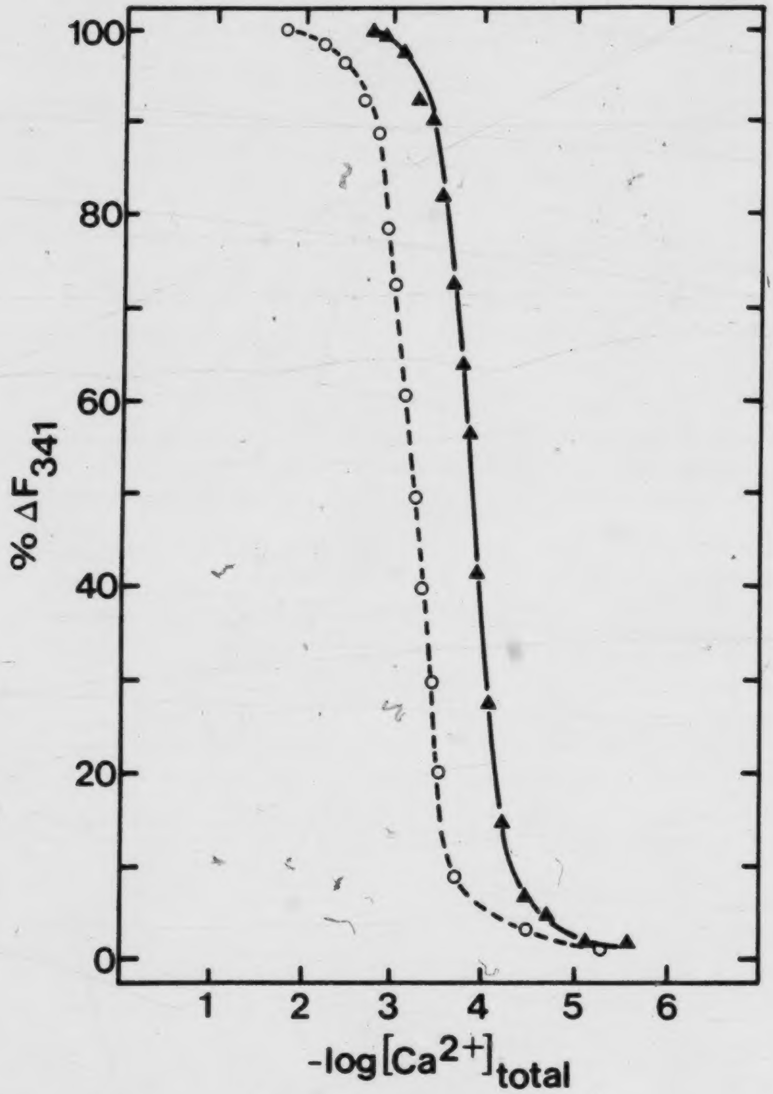

Fig. 17 Fluorescence titration of the α-subunit of S-100a protein at pH 7.5. The percentage change in fluorescence intensity is plotted as a function of the negative logarithm of total amount of Ca^{2+} added to the protein, at a concentration of 5.9 μM, in 0.1 M Tris/HCl containing 15 mM 2-mercaptoethanol at 20 °C, in the absence of (——), and in the presence of 90 mM KCl (------). The protein was excited at 282 nm and the emission was monitored at 341 nm.

TABLE X

Apparent Dissociation Constants for Ca^{2+} Obtained from Fluorescence Titrations of the α-subunit of S-100a Protein in 0.1 M Tris/HCl, 15 mM 2-mercaptoethanol at pH 7.5 and 8.4

		Apparent $K_{d\ Ca^{2+}}$
pH 7.5	buffer	$1.58 \pm .25 \times 10^{-4}$ M
	buffer + 90 mM KCl	$6.30 \pm .25 \times 10^{-4}$ M
	buffer + 3 mM Mg^{2+}	$1.62 \pm .25 \times 10^{-4}$ M
	buffer + 9.9 μM Zn^{2+}	$1.48 \pm .25 \times 10^{-4}$ M
pH 8.4	buffer	$5.60 \pm .10 \times 10^{-5}$ M $1.88 \pm .10 \times 10^{-4}$ M
	buffer + 90 mM KCl	$4.47 \pm .10 \times 10^{-4}$ M
	buffer + 9.9 μM Zn^{2+}	$7.07 \pm .10 \times 10^{-5}$ M

The excitation wavelength was at 282 nm with the emission being monitored at 341 nm. The concentration of the protein was 5.7 μM.

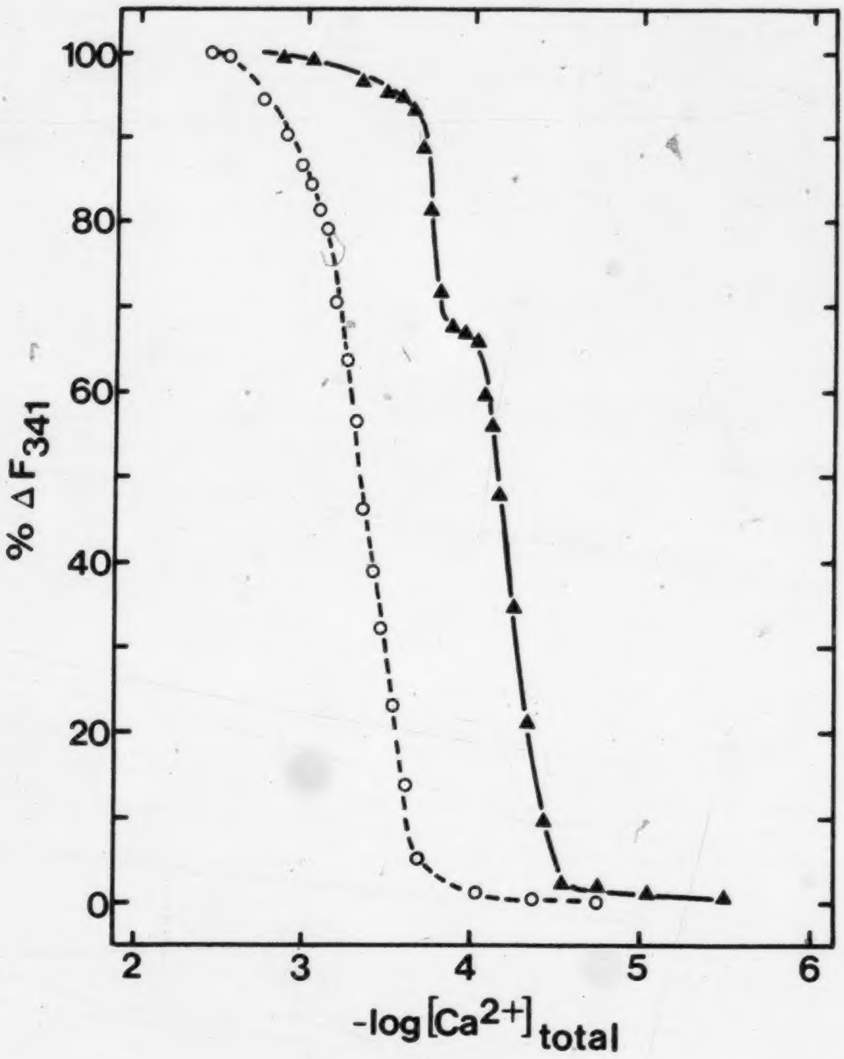

Fig. 18 Fluorescence titration of the α-subunit of S-100a protein at pH 8.4. The percentage change in fluorescence intensity is plotted as a function of the negative logarithm of the total amount of Ca^{2+} added to the protein at a concentration of 5.7 μM, in 0.1 M Tris/HCl containing 15 mM 2-mercaptoethanol at 20 °C, in the absence of (——), and in the presence of 90 mM KCl (------). The protein was excited at 282 nm and the emission was monitored at 341 nm.

from which an apparent $K_{dCa^{2+}}$ of 4.47 ± 0.1 x 10^{-4} M could be extrapolated. The monophasic curve implies that in the presence of KCl, only one class of Ca^{2+} binding site exists on the α-subunit, suggesting that K^+ can compete with Ca^{2+} for binding to the high affinity site. Alternatively, K^+ binding may induce a subtle conformational change in the environment near the high affinity site, thereby lowering the affinity of the protein for Ca^{2+}, and resulting in a lower K_d value (10^{-4} M). Baudier *et al.* (1986) reported that the affinity of S-100b for Ca^{2+} increased by approximately an order of magnitude in the presence of Zn^{2+}. Moreover, since they demonstrated that the α-subunit bound 3 mole $Zn^{2+}/\alpha\alpha$, the Ca^{2+} titration was repeated under similar conditions. At pH 8.4, the Ca^{2+} titration curve obtained in the presence of 3.55 mole $Zn^{2+}/\alpha\alpha$ was monophasic (data not shown), in contrast to the biphasic curve described earlier when the fluorescence titration was performed in the absence of Zn^{2+} (Fig. 18). However, upon comparing the Ca^{2+} concentration required to produce a half maximal change in the observed increase in the fluorescence intensity (which serves as an estimate of the affinity of the protein for Ca^{2+}), it was revealed that the 2 curves exhibited similar K_d values, which corresponded to 7.1 ± 0.1 x 10^{-5} M and 7.0 ± 0.1 x 10^{-5} M, respectively, in the presence and absence of Zn^{2+}. Taken together, these results suggest that Zn^{2+} binding to the α-subunit does not alter the affinity of the protein for Ca^{2+}. The failure to obtain a biphasic Ca^{2+} titration curve in the presence of Zn^{2+} could be due to the addition of large aliquots of Ca^{2+} during the titration and hence insufficient number of experimental points to differentiate between the 2 classes of Ca^{2+} sites which differ in their K_d values by half an order of magnitude.

It was reported earlier that at pH 8.4, Ca^{2+} induced a larger enhancement in the intrinsic fluorescence of the protein than that observed at pH 7.5. In order to examine the effect of pH on the fluorescence properties of S-100α, the protein was titrated with base in the presence and absence of Ca^{2+}. It can be seen in Fig. 19, that the fluorescence enhancement by Ca^{2+} was influenced by pH. Below pH 6, the effect was minimal but reached a maximum between pH 8 - 9 for the Ca^{2+} bound S-100α, upon excitation at 282

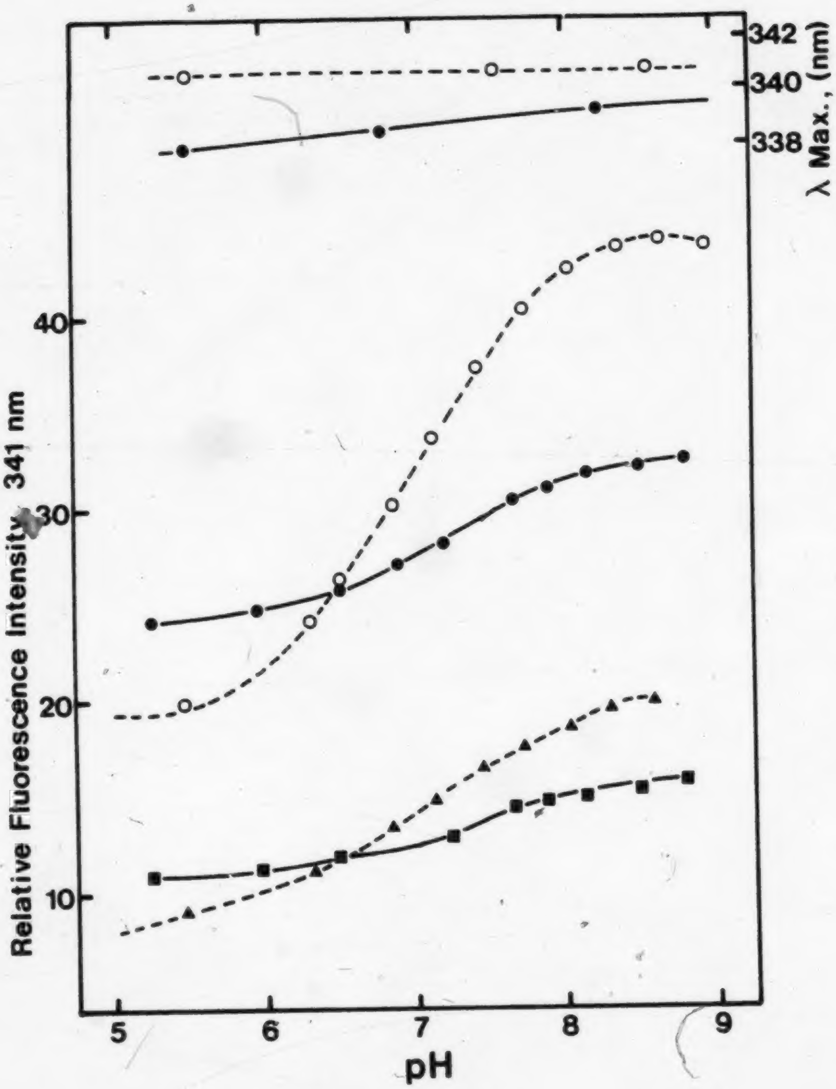

Fig. 19 Effect of pH on fluorescence intensity and emission maximum (λ_{max}) of the α-subunit of S-100a protein. The protein (6.6 μM) in a buffer containing 5 mM Mes, pH 5.5, 50 mM NaCl, and 5 mM 2-mercaptoethanol, was titrated with NaOH in the presence of 1 mM EDTA (——), and 1.4 mM Ca^{2+} (------). The excitation wavelength was at 282 nm (○ , ●) and at 295 nm (■ , ▲), while the emission was monitored at 341 nm.

nm (where both Trp and Tyr absorb) and at 295 nm (maximally exciting the Trp). On the other hand, the position of the emission maximum (λ_{max}) was not significantly affected by pH. With the metal free protein, a similar, albeit smaller fluorescence enhancement was also observed upon raising the pH. In both instances, the effect was reversible below pH 10 whether Ca^{2+} was present or not. From the shapes of these titration curves, it can be suggested that the Trp fluorescence of S-100α was affected by a chemical group(s) titrating with a p*K* of 7.05 which had a greater effect in the presence of Ca^{2+} than in its absence. Calissano *et al.* (1969) had also observed that the Ca^{2+} induced fluorescence enhancement in a mixture of S-100 protein was affected by some group titrating with a p*K* of 7.2, which conceivably could be a histidine residue since its p*K* value varies between 6 - 7.

4. Terbium Binding Properties of the α-subunit. Terbium has often been used as a Ca^{2+} analog to function as a reporter group for the Ca^{2+} binding site (Horrocks, 1982; Dockter, 1983). Since the α-subunit contains one EF hand type Ca^{2+} binding site/chain, the effect of Tb^{3+} on S-100α was also examined by a variety of spectroscopic techniques.

CD was employed to examine the effect of Tb^{3+} binding to the α-subunit. Typical far UV CD spectra of the α-subunit in 50 mM Pipes, 5 mM 2-mercaptoethanol pH 6.5, in the absence and presence of Tb^{3+} are shown in Fig. 20. Addition of 0.96 Tb^{3+}/subunit resulted in a 5.6% decrease in the $[\theta]_{222}$ value, from -16860 to -15909 ± 300 $deg.cm^2.dmol^{-1}$. Analysis of CD data by the method of Chen *et al.* (1974), indicated the α-helical content to decrease from 56% to 52% when Tb^{3+} binds to the α-subunit.

The effect of Tb^{3+} on the aromatic CD spectrum of the α-subunit at pH 6.5 is shown in Fig. 21. The addition of 0.96 Tb^{3+}/subunit resulted in a small perturbation of the tyrosine bands observed at 278 and 284 nm, suggesting that Tb^{3+} only induces a small alteration in the environment near the tyrosines.

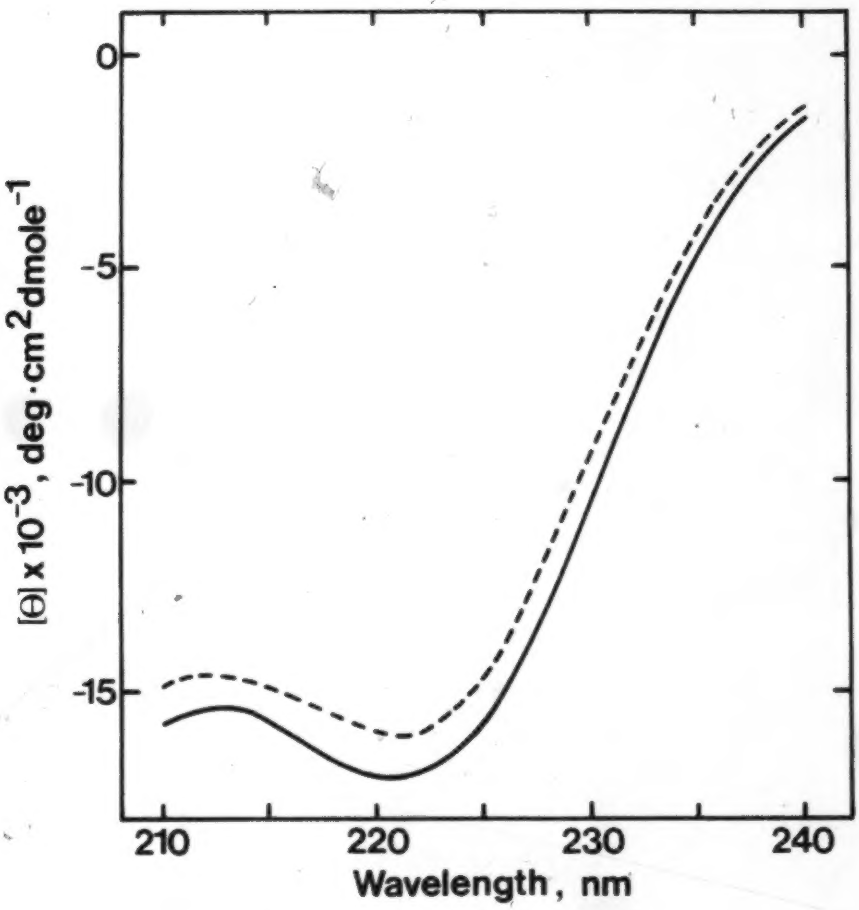

Fig. 20 Far UV CD spectra of the α-subunit of S-100a protein in the absence of (——), and in the presence (------) of 26 µM Tb^{3+} (1 mol / Tb^{3+} mole α-chain). The solvent system was 50 mM Pipes, pH 6.5, containing 5 mM 2-mercaptoethanol.

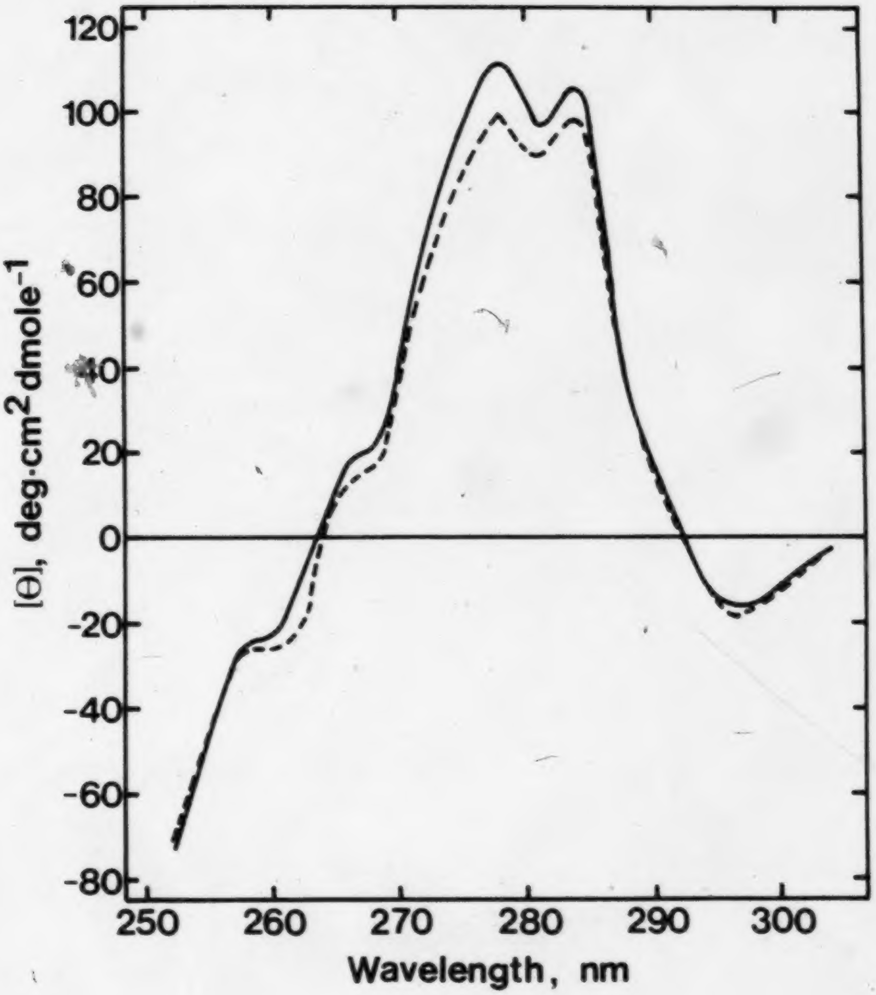

Fig. 21 Near UV CD spectra of the α-subunit of S-100a protein in 50 mM Pipes, pH 6.5, containing 5 mM 2-mercaptoethanol, in the absence of Tb^{3+} (——), and in the presence of 1 Tb^{3+} / α-subunit (------). The concentration of the protein was 62.3 μM.

The effect of Tb^{3+} on the local environment of aromatic residues was further examined by UV difference spectroscopy. In Fig. 22, it can be seen that addition of 0.52 mole Tb^{3+}/mole subunit resulted in a blue shift in the tyrosine absorption bands at 277 and 284 nm as well as the tryptophan band at 292.5 nm. The blue shifts suggested that Tb^{3+} induced the aromatic residues corresponding to these absorption bands to become more exposed to solvent (Donovan, 1973). Alternatively, the small change in the $\Delta\varepsilon_{284}$ value of -31 could be interpreted as an electrical charge induced change as observed in ribonuclease where the $\Delta\varepsilon_{287}$ value was of the order of 60 (Donovan, 1965). Although the magnitude of the difference peaks induced by Tb^{3+} was small, the effect was significant since the standard deviation of the $\Delta\varepsilon$ value was $\pm$ 4.8 M^{-1} cm^{-1}. Moreover, when Ca^{2+} was added to the apoprotein under identical conditions (i.e. 0.5 Ca^{2+}/α-chain), no UV difference spectrum was observed (data not shown), suggesting that Tb^{3+} was more effective than Ca^{2+} in perturbing the environment near the aromatic residues.

Terbium binding to S-100α was also investigated by fluorescence spectroscopy. Excitation of terbium from the ground level ($^7F_6$) to the luminescence level ($^5D_4$), can be achieved either by direct excitation at its major absorption band at 219 nm (Horrocks, 1982), or indirectly via excitation of adjacent aromatic chromophores by the process of energy transfer (Crosby and Whan, 1962). Upon excitation of the α-subunit at 274 nm (maximally exciting tyrosines) the emission maximum was centered at 338 ± 1 nm due to the presence of the single tryptophan. Addition of 1.06 mole Tb^{3+}/α-subunit resulted in no significant change in the position of the emission maximum nor fluorescence intensity (Fig. 23). However, in the region of the emission spectrum with wavelength longer than 400 nm, a significant increase in fluorescence intensity was observed with peaks detected at 489 ± 1, 543 ± 1 and 581 ±1 nm (Fig. 23). For instance, at 543 nm, the fluorescence intensity increased by 37 fold upon the addition of Tb^{3+} (Table XI). The presence of these peaks was most likely due to sensitized emission from terbium bound to the protein since with identical instrument settings employed, free Tb^{3+} in buffer did not give rise to any peaks in

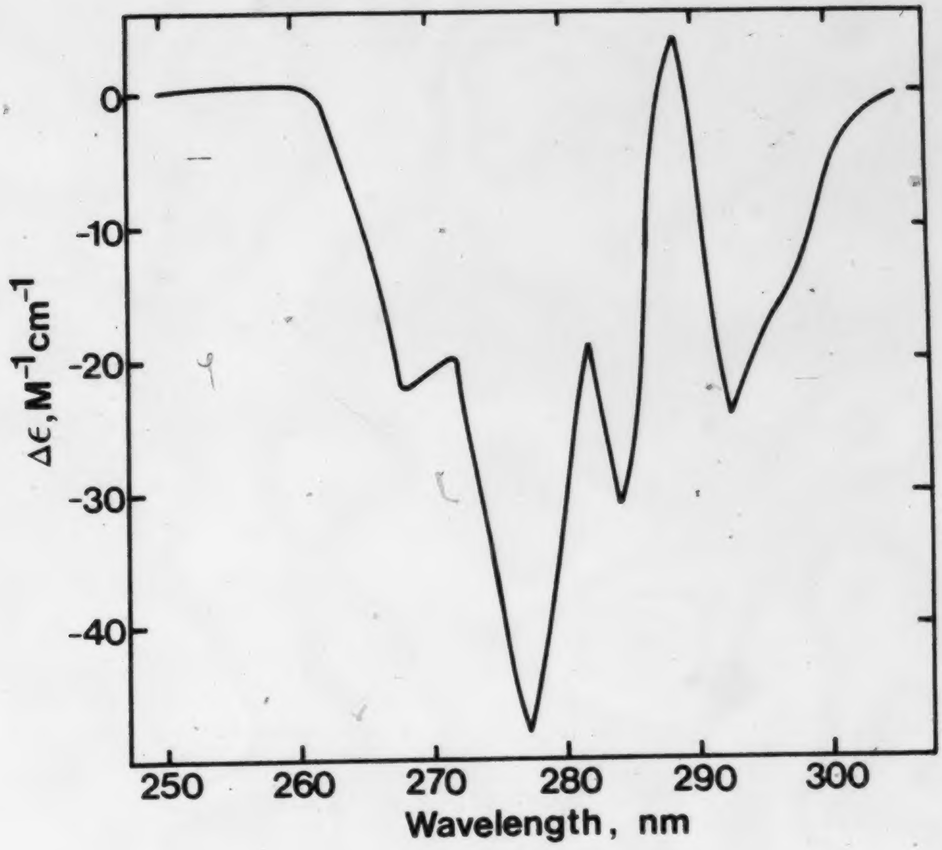

Fig. 22 UV difference spectrum induced by the addition of Tb^{3+} to the α-subunit of S-100a protein at a Tb^{3+} / α-subunit molar ratio of 0.52 : 1. The protein (42.4 μM) was in a solvent system containing 50 mM Pipes, pH 6.5. The standard deviation was ± 4.8 M^{-1} cm^{-1}.

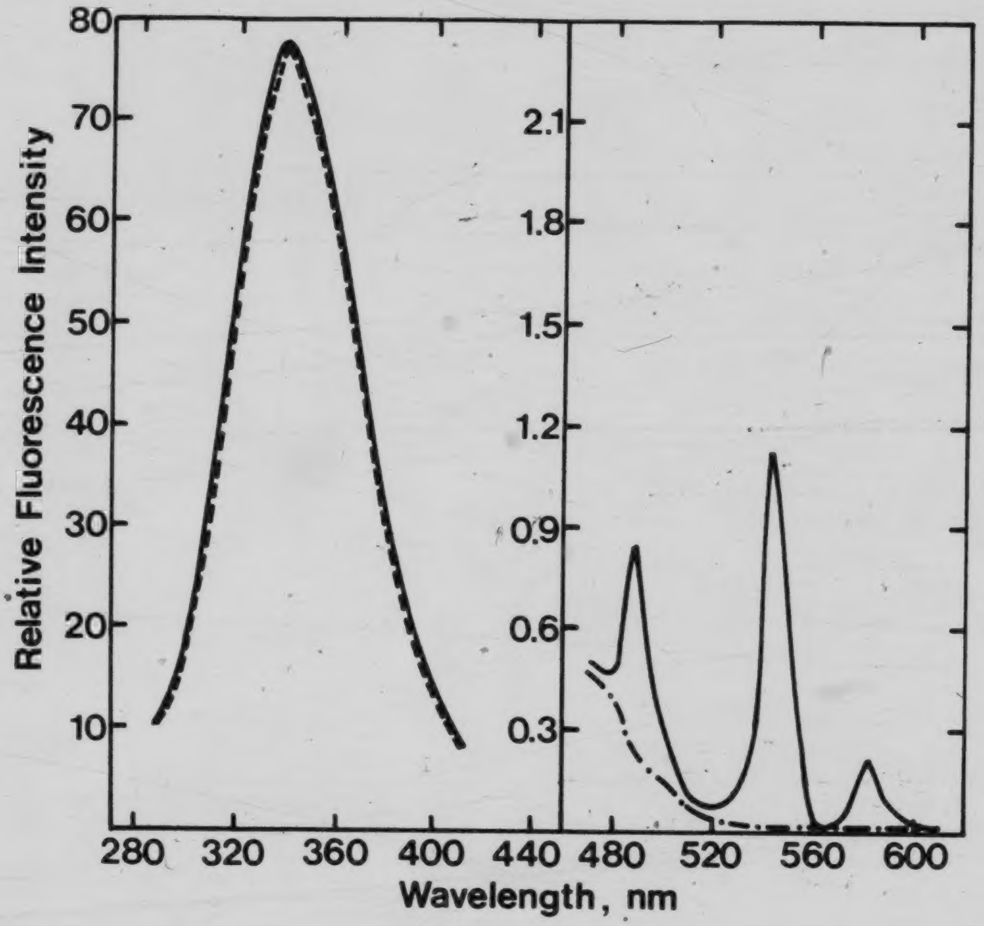

Fig. 23 Fluorescence spectra of the α-subunit of S-100a protein (4.3 μM) in 50 mM Pipes, pH 6.5 and 5 mM 2-mercaptoethanol, in the absence of (------) and in the presence (——) of 1.06 mol Tb^{3+} / α-subunit. In the 480 - 600 nm region, (— • — •) represents the emission spectrum of buffer containing Tb^{3+} at a concentration identical to that present in the protein sample.

TABLE XI

Effect of Tb^{3+} Addition on Emission Spectra of S-100α in 50 mM Pipes, 5 mM 2-mercaptoethanol at pH 6.5

λ_{ex} (nm)*	λ_{em} (nm)*	RF ($-Tb^{3+}$)**	RF ($+Tb^{3+}$)**	ΔF, (%)
295	338	23.20	23.51	1.3
	489	0.29	0.68	2.4
	543	0.05	1.10	27.5
	581	0.03	0.22	7.3
274	338	78.00	78.5	0.6
	489	0.20	0.8	4.0
	543	0.03	1.1	36.7
	581	0.02	0.2	10.0

* λ_{ex} represents the excitation wavelength employed in the experiment and λ_{em} represents the wavelength where peaks were observed in the emission spectra.

**RF denotes the relative fluorescence intensity in arbitrary units at the wavelength indicated.

ΔF denotes percentage change in the observed fluorescence intensity before and after addition of Tb^{3+} equivalent to 1.05 mole Tb^{3+}/subunit.

The concentration of the protein was 4.5 μM.

this region of the emission spectrum (Fig. 23). Terbium binding to the α-subunit was also studied by maximally exciting the tryptophan at 295 nm. Again, Tb^{3+} had no significant effect on the intrinsic fluorescence properties of the protein (Table XI). However, peaks due to sensitized Tb^{3+} emission were observed in the 480 - 600 nm region. Collectively, these data suggest that resonance energy transfer from one or more aromatic residue donor(s) to the protein bound Tb^{3+}, is responsible for the observed luminescence properties of this metal ion. The exact identity of the donor is not known even though the observation of an excitation maximum at 274 nm (when emission was set at 543 nm) suggests tyrosine is involved in this process. However, the involvement of tryptophan cannot be ruled out since excitation of the Tb^{3+} protein at 295 nm resulted in sensitized Tb^{3+} emission with intensity similar to that observed when the λ_{ex} was set at 274 nm (Table XI).

Terbium titration of the α-subunit was performed by monitoring the fluorescence enhancement of Tb^{3+} emission at 543 nm. Addition of 1 mole Tb^{3+}/ mole α-subunit resulted in a 40% increase in the fluorescence intensity. However, when the titration was continued beyond 1:1 ratio, an increase in turbidity was observed, suggesting the protein underwent aggregation or precipitation. Hence, we were unable to obtain a K_d value of the α-subunit for Tb^{3+} using this technique.

The stoichiometry of Tb^{3+} binding to S-100α was examined by an ultrafiltration partition technique similar to that described for Ca^{2+} binding assays. When Tb^{3+} at a molar ratio of 1.3 and 1.55/subunit was added to the protein, the numbers of Tb^{3+} bound per subunit were 0.72 ± .30 and 0.92 ± 0.2, respectively. In these studies, an upper limit of 1.5 fold molar excess of Tb^{3+} per α-chain was used since fluorescence studies suggested that the protein precipitated upon exceeding a 2.3:1 ratio.

Conventional sedimentation equilibrium studies performed with Tb^{3+} bound protein at a molar ratio of 1.5:1 gave a weight average molecular weight ranging from 20,000 to 26,000 ± 1000, suggesting that Tb^{3+} induced aggregation of the protein to a small extent.

C. Denaturation Studies of the α-subunit

1. Thermal Denaturation. Thermal denaturation studies were performed with S-100α in 25 mM Pipes, 100 mM NaCl, 1 mM EDTA and 15 mM 2-mercaptoethanol, at pH 7.1. The unfolding of the protein was monitored by following the decrease in the molar ellipticity value at 222 nm, $[\theta]_{222}$, as a function of temperature, as shown in Fig. 24. It can be seen that the apoprotein is relatively stable to thermal denaturation as the $[\theta]_{222}$ value decreased from -17540 to -15650 ± 300 deg.cm^2.dmol^{-1}, when the temperature was raised from 4.6°C to 50.1°C. The unfolding process continued as the temperature was increased to 71°C, resulting in the $[\theta]_{222}$ value decreasing by another 13.5% . At 78°C, the highest temperature attainable using a circulating water bath, the final $[\theta]_{222}$ value observed was -11567 ± 300 deg.cm^2.dmol^{-1}, suggesting the protein still retained 68% of secondary structure relative to the native protein. The spectra recorded upon cooling the sample were essentially similar to those observed for the native protein, suggesting that the unfolding process was reversible.

2. Denaturation by Guanidine Hydrochloride. Since thermal denaturation failed to completely unfold the α-subunit under native solvent conditions, the stability of the α-subunit was examined by using GdnHCl as a denaturant. The denaturation studies were performed in 25 mM Mops, 100 mM NaCl, 2 mM DTT, pH 7.1 at 25°C and in the absence and presence of Ca^{2+}. Far UV CD was employed to monitor the unfolding of the protein and the data were then normalized and expressed as fractional conformational change, f, as shown in Fig. 25. From the denaturation curves, it can be seen that in the presence of 5 M GdnHCl the apoprotein has undergone extensive unfolding, resulting in the $[\theta]_{221}$ value dropping from -16632 to -815 ± 300 deg.cm^2.dmol^{-1}. Conventional sedimentation equilibrium experiments performed under these conditions gave a weight average molecular weight of 10,850 ± 1000, suggesting the α-subunit exists as a monomer (data not shown). In the presence of Ca^{2+}, extensive

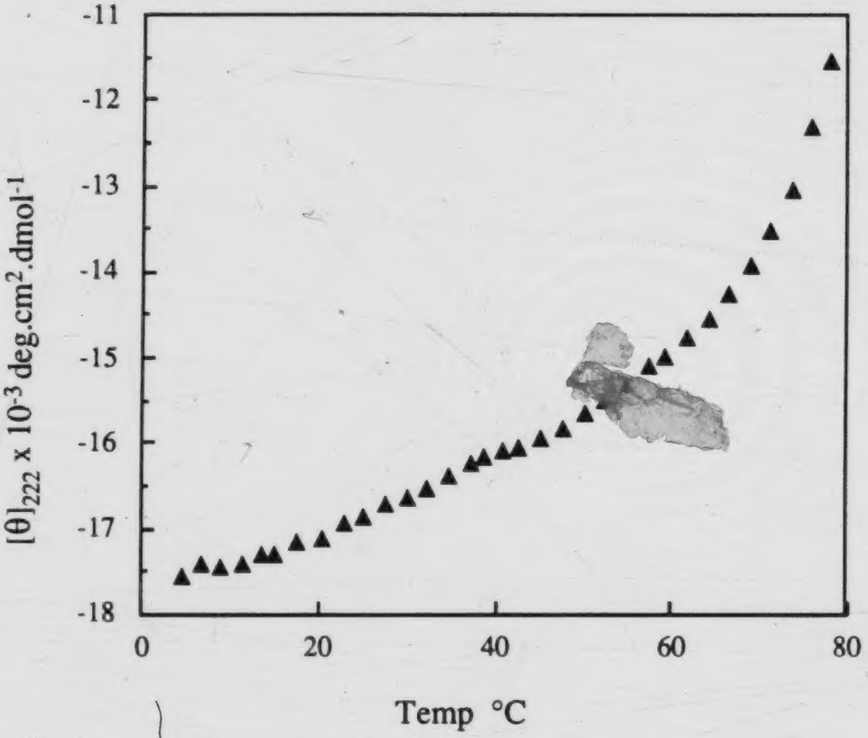

Fig. 24 Thermal denaturation of the α-subunit of S-100a protein in 25 mM Pipes, 100 mM NaCl, 1 mM EDTA, 15 mM 2-mercaptoethanol at pH 7.1. The unfolding of the protein was monitored by following the decrease in the ellipticity value at 222 nm.

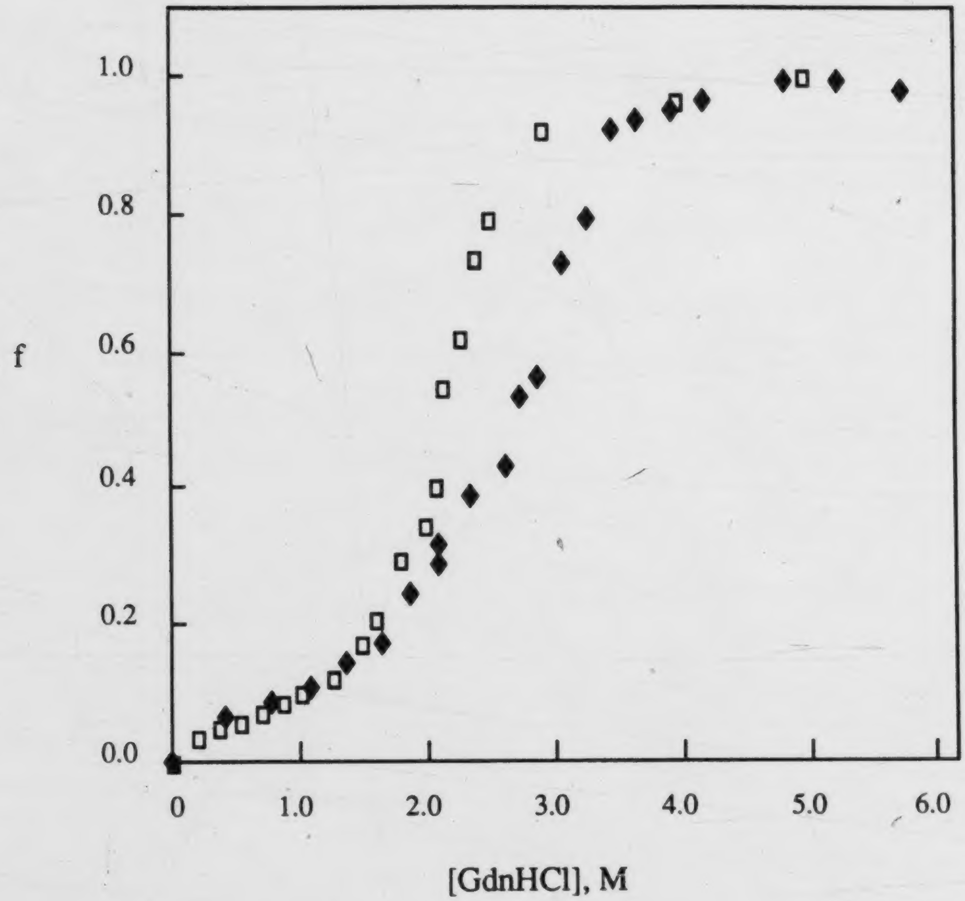

Fig. 25 Fractional conformational change, f, of the α-subunit of S-100a protein in 25 mM Mops, 0.1 M NaCl, 2 mM DTT, containing 1 mM EDTA (□), or 2 mM Ca^{2+} (◆), as a function of the concentration of GdnHCl at 25 °C. The unfolding of the protein was monitored by following the decrease in $[\theta]_{221}$ values.

unfolding of S-100α was also observed with the final $[\theta]_{221}$ value similar to that observed for the apoprotein. Moreover, both denaturation curves were reversible and exhibited a single step transition, suggesting that the unfolding of the protein involved a 2 step mechanism. A comparison of the 2 denaturation curves showed that the transition region observed for the Ca^{2+} bound protein appeared to occur over a slightly wider range of GdnHCl concentration, 1.75 to 3.5 M, as opposed to a sharper transition of 1.6 to 3 M for the apoprotein. Moreover, the denaturant concentration required to induce a 50% change in the conformation was also higher in the presence of Ca^{2+}, 2.65 M versus 2.15 M for the apoprotein. Taken together, these results suggest that binding of Ca^{2+} to the α-subunit increases the stability of the protein against denaturation.

D. Sedimentation Equilibrium studies of the α-subunit

Conventional sedimentation equilibrium studies were performed in order to obtain the weight average molecular weight of S-100α under denaturing as well as native solvent conditions. In the latter, the buffer system consisted of 50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT or 5 mM 2-mercaptoethanol, pH 7.5. In some of the analytical runs, Ca^{2+} or Zn^{2+} was added to the solvent system to examine its effect on the sedimentation behaviour of S-100α.

Since previous denaturation studies suggested that GdnHCl caused extensive unfolding of S-100α, sedimentation equilibrium runs were performed in 6 M GdnHCl, 50 mM Tris/HCl, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, pH 7.5. A representative result is presented graphically as a plot of the natural logarithm of the protein concentration expressed as absorption units versus the square of the distance from the center of rotation (Fig. 26). The plot was essentially linear and provided an estimate of the weight average molecular weight over the whole cell, which was found to be 11,020 ± 1000. This value is similar to that calculated from the amino acid sequence of the α-subunit (M_r = 10,400, Isobe and Okuyama, 1981a). When the sedimentation study was repeated in the absence of

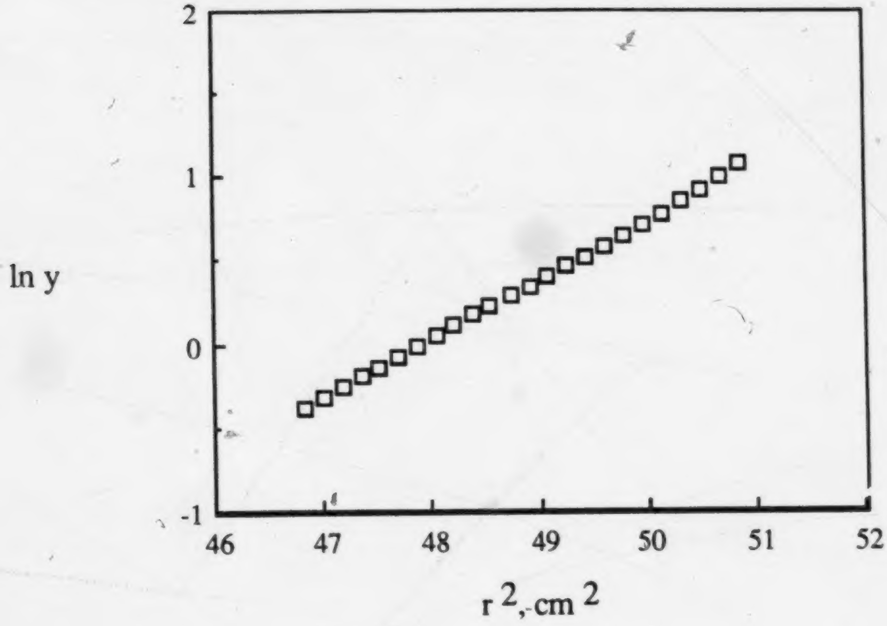

Fig. 26 Molecular weight study of the α-subunit of S-100a protein in 6 M GdnHCl, 50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5, using the conventional sedimentation equilibrium technique. The result is depicted as a plot of ln y versus r^2. An initial protein concentration of 0.4 mg/ml and a rotor speed of 34,000 rpm was used.

DTT, a similar result was obtained , indicating that the molecular weight of the α-subunit is not affected by the oxidation state of the sulfhydryl group.

Sedimentation equilibrium experiments were also performed under native solvent conditions. In the absence of metal ions, at initial loading concentrations of 2.7 and 1.6 mg/ml, the point weight average molecular weight did not vary significantly with the protein concentration across the cell (Fig. 27). Plots of the natural logarithm of the concentration (ln y) versus r^2, were essentially linear (not shown) which gave weight average molecular weights of 21,370 ± 1000 and 19,580 ± 1000 respectively, at these two protein concentrations. When the sedimentation experiments were performed in the presence of 1 mM Ca^{2+}, a weight average molecular weight distribution ($\overline{M}_w$) ranging from 19,000 to 24,000 ± 1000 was observed across the cell with a sample of an initial loading concentration of 1.5 mg/ml (Fig. 28). When the run was repeated at a lower protein concentration of 0.36 mg/ml the $\overline{M}_w$ ranged from 19,000 to 26,000 ± 1000. In the presence of 0.2 mM Zn^{2+}, the $\overline{M}_w$ distribution obtained from experiments performed with protein at initial loading concentrations of 1.6 and 0.56 mg/ml, varied from 18,000 to 25,000 ± 1000 (Fig. 29). Hence, these sedimentation equilibrium results suggest that both Ca^{2+} and Zn^{2+} caused the α-subunit to aggregate to a small extent. In these sedimentation studies, there was some indication that the molecular weight of S-100α showed a concentration dependence when the protein concentration was less than 1 mg/ml. This suggests that the homodimer may dissociate to give monomeric subunit at low protein concentration.

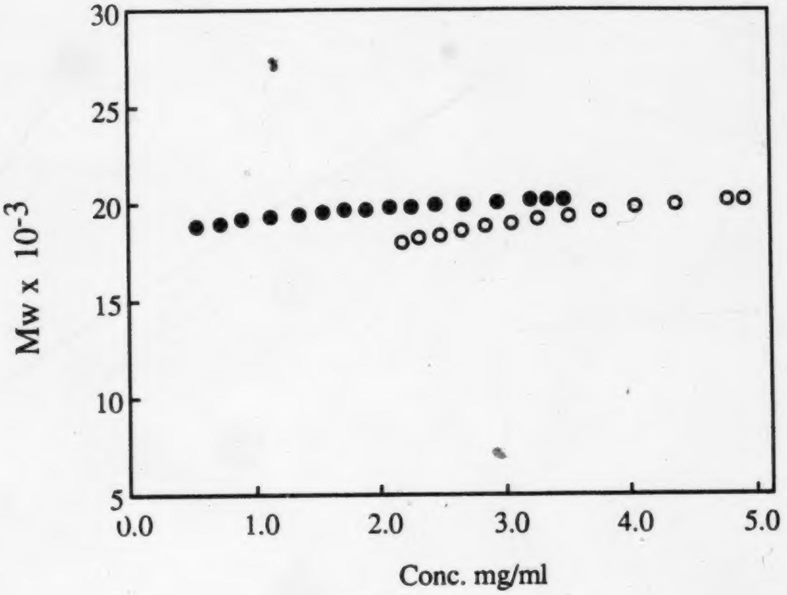

Fig. 27 Molecular weight of the α-subunit of S-100a protein as a function of the protein concentration in the absence of metal ions. Conventional sedimentation equilibrium experiments were performed in 50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA and 1 mM DTT, pH 7.5. Initial protein concentrations and equilibrium rotor speeds were: (○) 2.7 mg/ml operating at a rotor speed of 15,000 rpm, and (●) 1.6 mg/ml operating at a rotor speed of 18,000 rpm.

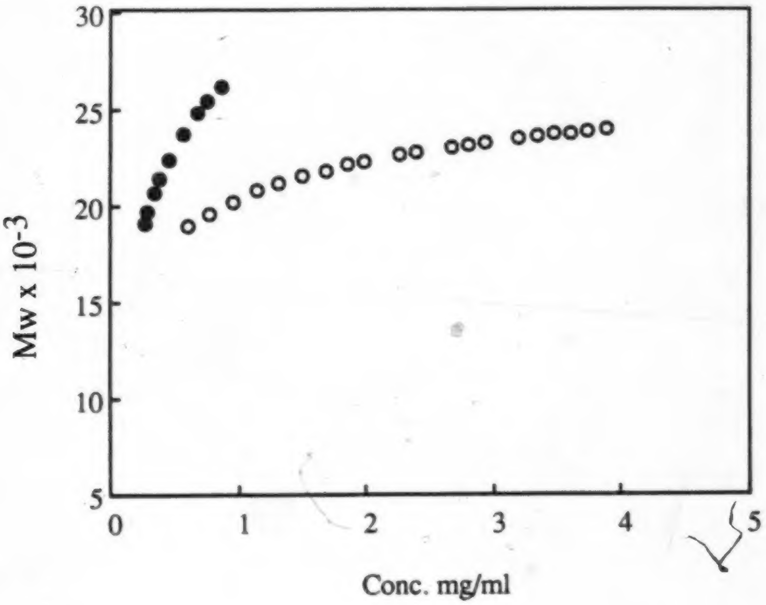

Fig. 28 Molecular weight of the α-subunit of S-100a protein as a function of protein concentration in the presence of Ca^{2+}. Conventional sedimentation experiments were performed in 50 mM Tris/HCl, 100 mM NaCl, 1 mM DTT and 1 mM Ca^{2+}, pH 7.5. Initial protein concentration was: (○) 1.5 mg/ml, and (●) 0.77 mg/ml, operating at a rotor speed of 15,000 rpm.

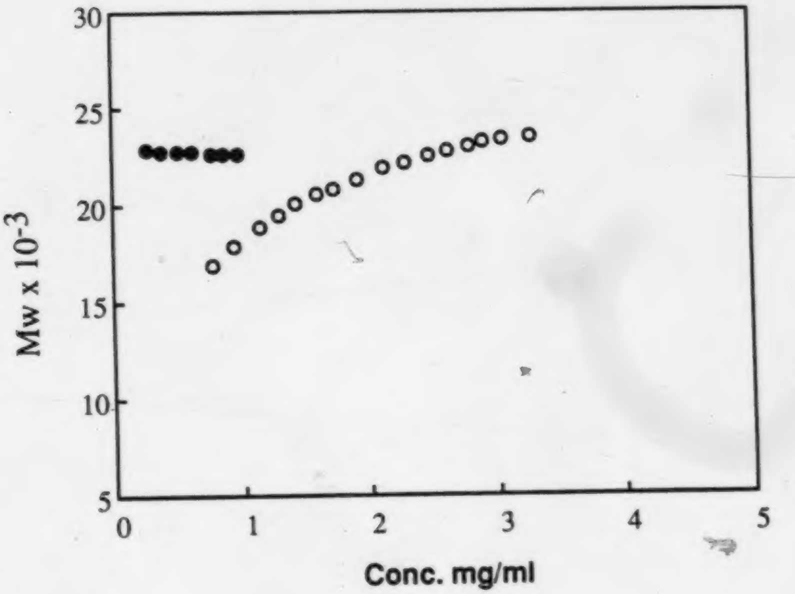

Fig. 29 Molecular weight of the α-subunit of S-100a protein as a function of protein concentration in the presence of Zn^{2+}. Conventional sedimentation equilibrium experiments were conducted in 50 mM Tris/HCl, 100 mM NaCl, 5 mM 2-mercaptoethanol and 0.2 mM Zn^{2+}, pH 7.5. Initial protein concentration was: (○) 1.63 mg/ml, and (●) 0.56 mg/ml, operating at a rotor speed of 15,000 rpm.

E. Discussion

The isolation of the α-subunit of S-100a protein has allowed the characterization of its physico-chemical and metal ion binding properties. These studies have demonstrated that CD, UV difference and fluorescence spectroscopy are useful techniques for monitoring the conformational changes induced by metal ion binding to the α-subunit. The ellipticity values reported in this study for the far UV CD spectra were slightly lower than those described earlier (Leung *et al.*, 1986) but the magnitudes of the conformational changes induced by the addition of metal ions were similar. The discrepancy is most likely due to different batches of d-10 camphor sulfonic acid used in the calibration of the instrument. Nevertheless, the results obtained from these studies have enabled us to compare the metal ion binding properties of the α-subunit with those of the β-subunit that exist in the S-100a heterodimer and in the S-100b homodimer.

In general the S-100 protein undergoes a conformational change upon binding Ca^{2+} and Zn^{2+} but subtle differences exist in the manner in which the aromatic chromophores are being perturbed. For instance, with S-100a, Ca^{2+} induced the tryptophan and one or more tyrosine and phenylalanine residues to become more exposed to solvent, whereas in S-100b, the tyrosine and phenylalanine residues are buried (Mani and Kay, 1983a; Mani *et al.*, 1983; Baudier and Gerard, 1983). Zn^{2+} binding also resulted in gross conformational changes in S-100a whereas S-100b is not affected (Mani and Kay, 1983b)). The changes induced in the environment of the aromatic amino acid chromophores by Ca^{2+} and Zn^{2+} are the same in S-100b but different in S-100a.

(i) Ca^{2+} binding properties

Ca^{2+} induced a conformational change in the α-subunit similar to that observed with S-100a and S-100b, namely a decrease in the α-helical content. Analysis of the far UV CD data obtained at pH 7.5, according to the method of Provencher and Glöckner (1982), revealed that in addition to a 7% reduction in the α-helical content, Ca^{2+} also induced a 21% increase in the β-sheet content. Near UV CD spectroscopy revealed that the CD signals, due to the tyrosines (at 278 and 284 nm) in the α-subunit, are approximately 2.5 times larger than those of S-100a protein. The higher ellipticity values observed for S-100α could be due to intramolecular interaction between the 2 tyrosine residues. Strickland (1972) reported that a coupling in the vibrational energy levels of 2 tyrosines in close proximity to each other, contributed to the intense tyrosyl CD bands of ribonuclease. In spite of these differences, the effect of Ca^{2+} on the microenvironment of aromatic residues in the α-subunit is more akin to that in S-100a protein, but subtle differences do exist in the extent of these perturbations. In both proteins, Ca^{2+} induced the exposure of a single tryptophan, and one or more tyrosine and phenylalanine residues to solvent; however, a comparison of their respective values in the UV difference spectra at pH 7.5 revealed that these residues are in a more polar environment in the α-subunit than in S-100a. For instance, the Δε values at 284 nm for the Ca^{2+} induced spectra of the α-subunit is -986 $M^{-1}cm^{-1}$ (Table VIII) compared to -650 M^{-1} cm^{-1} for S-100a (Mani and Kay, 1983a). This suggests that the Ca^{2+} effect on the aromatic chromophores in the α-subunit in S-100a is modified by the presence of the β-chain, in which the tyrosine and phenylalanine residues become buried, resulting in a lower Δε value at the corresponding wavelength for S-100a compared to the α-subunit. In the case of the α-subunit, the observed $\Delta\varepsilon_{284.5}$ value of -986 $M^{-1}cm^{-1}$ is lower than the $\Delta\varepsilon_{287}$ value of -700 $M^{-1}cm^{-1}$ reported for the complete exposure of a buried phenolic group to solvent (Donovan, 1973). However, the fact that the absorption band of tryptophan overlaps with that of tyrosine in the 280 nm region, suggested that Trp could contribute to the magnitude of the large tyrosine difference peak observed at 284.5 nm. When the effect due to Trp has been accounted for, this would result in a less negative Δε value, implying that Ca^{2+} binding to the α-subunit is likely to affect the environment of only one of the two tyrosines present in the protein.

Fluorescence titrations with S-100α confirmed the findings of the binding stoichiometry studies whereby 2 Ca^{2+} binding sites are found in the protein at pH 8.4. The apparent K_d values of the 2 Ca^{2+} binding sites, obtained from fluorescence titration are $1.88 \pm 0.1 \times 10^{-4}$ M and $5.60 \pm 0.1 \times 10^{-5}$ M. At pH 7.5, the protein possesses only one Ca^{2+} binding site with a K_d value of $1.58 \pm 0.25 \times 10^{-4}$ M. Hence, at both pH values, the α-subunit binds Ca^{2+} with affinities similar to those observed with S-100a and S-100b protein (Mani *et al.*, 1983; Mani and Kay, 1983a). At pH 8.4, although fluorescence titration of the α-subunit yielded a biphasic curve (Fig.18), compared to the monophasic curve obtained from CD (Fig. 13), the discrepancy in the shapes of the titration curves could be due to differences in the protein concentration used in the 2 techniques. The fluorescence titration experiments were generally performed with dilute protein solutions containing ~ 6×10^{-6} M of α-subunit. As a result of the low protein concentration, a Ca^{2+} : protein molar ratio of 1:1 could be achieved after the addition of a few aliquots of a $CaCl_2$ stock solution. From the titration curve (Fig. 18), it can be seen that further addition of Ca^{2+} beyond the 1:1 ratio resulted in an increase in the tryptophan fluorescence. Hence, by monitoring the change in fluorescence intensity as a function of the amount of Ca^{2+} added, one can detect the conformational change associated with Ca^{2+} binding to the high affinity site ($K_d \sim 10^{-5}$ M), as well as the low affinity site ($K_d \sim 10^{-4}$ M) present on the α-subunit at pH 8.4. On the other hand, with the CD titrations which were performed with the α-subunit at a concentration of 8.7×10^{-5} M, no significant change in the ellipticity value could be observed until a Ca^{2+} : protein ratio of 0.53:1 was attained (Fig. 13). As a result of the high protein concentration used in the experiment, CD spectroscopy was slower, compared to fluorescence, in sensing the conformational change induced by Ca^{2+} binding to the high affinity site which occurs in the early part of the titration. Hence, the K_d value obtained from the CD titration probably reflects the average affinity of the 2 Ca^{2+} binding sites. On the other hand, the higher protein concentration used in the CD experiment, allowed the detection of cooperativity between the 2 Ca^{2+} binding sites, as suggested from a Scatchard analysis of the titration data.

The Ca^{2+} binding properties of the α-subunit of bovine brain S-100a protein have also been studied by Baudier *et al.* (1986), Baudier and Gerard, (1986) and Masure *et al.* (1984). These authors find that the conformational changes induced by Ca^{2+} binding to the α-subunit are similar to those reported in our study. However, there are discrepancies with regards the binding stoichiometry. We have reported that at pH 7.5 and 8.4, 1 and 2 Ca^{2+} binding sites exist on each α-chain, respectively. In contrast, Baudier *et al.* (1986), using a continuous flow dialysis technique, reported the presence of 4 Ca^{2+}/mol protein dimer (or 2 Ca^{2+}/monomer) at both pH values. The reason for the discrepancy in the data observed at pH 7.5 is not known, but may reflect differences in experimental conditions. The Ca^{2+} binding experiments in our study were performed with Tris/HCl buffer at a physiological ionic strength of 100 mM and at a protein concentration of 165 μM as opposed to their experimental conditions of 20 mM Tris/HCl and a protein concentration of 30 μM. In this respect, our results for the binding stoichiometry experiments are similar to those reported by Calissano *et al.* (1974). Equilibrium dialysis experiments performed by the latter group at pH 8.3, using $^{45}Ca^{2+}$, revealed the presence of 4 Ca^{2+} binding sites per 21,000 molar mass (g/mol), whereas at pH 7.6, there was evidence for 2 Ca^{2+} binding sites per S-100 dimer. Furthermore, in addition to the differences in the Ca^{2+} binding stoichiometry, the various preparations of S-100α from these laboratories also exhibited different affinities for Ca^{2+}. A comparison of our apparent K_d values for Ca^{2+} obtained from fluorescence titration at pH 7.5, with that of Masure *et al.* (1984), revealed that the latter group had reported a lower value of 7×10^{-5} M for their preparation of S-100α when the titration was performed in 50 mM Tris/HCl. The difference in the affinity could be due to the effect of the ionic strength of the buffer used in these experiments, since Baudier *et al.* (1986) reported that increasing the ionic strength of Tris/HCl buffer resulted in an antagonistic effect on Ca^{2+} binding to S-100a and S-100b. Conceivably, the high concentration of Tris cation may lower the affinity of the protein for the Ca^{2+} cation.

A comparison of the amino acid sequence of the bovine α and β subunits of S-100 protein (Isobe and Okuyama, 1978, 1981a) with the sequences of other intracellular calcium binding proteins (CaBP), showed that the α and β subunits belong to the subclass of 2 Ca^{2+} binding domains CaBP such as parvalbumin (Gerday, 1976) and intestinal calcium binding protein (ICaBP) (Fullmer and Wasserman, 1981). Amongst this subgroup, the S-100 protein has been predicted to closely resemble ICaBP in conformation (Szebenyi *et al.*, 1981). The three dimensional structure of bovine ICaBP revealed that an EF hand type Ca^{2+} binding site exists in the C-terminus of the protein, while a pseudo invariant site containing a 2 amino acid insertion in the loop, is found at the N-terminus (Szebenyi *et al.*,1981; Szebenyi and Moffat, 1986). The 3 dimensional structure of the S-100 protein is not known but an EF hand Ca^{2+} binding site had been predicted at the C-terminus (residues 62-73 on the α-chain and residues 61-72 on the β-chain) (Isobe and Okuyama, 1978; 1981a). In addition, a second pseudo invariant site has been proposed to occur at the N-terminus of both subunits (at residues 19α-32α) (Szebenyi *et al.*, 1981). Moreover, the 2 subunits have identical ligands at the C-terminal Ca^{2+} binding site but differ in the N-terminal site at the position corresponding to calcium ligand -X. Irrespective of the fact that both proteins share a similar arrangement of 1 variant and 1 EF hand site, the S-100 protein, which binds 2 Ca^{2+}/ subunit at pH 8.4 with K_d values of ~ 10^{-4} and 10^{-5} M, exhibits a lower affinity for Ca^{2+}, compared to ICaBP which binds 2 Ca^{2+} with pK_d values of ~ 6 (Wasserman and Fullmer, 1982). Szebenyi and Moffat (1986) suggested that a lack of sequence homology in the N-terminal region of the S-100 protein with ICaBP, may have a role in influencing the affinity of the S-100 protein for Ca^{2+}. The absence of a proline residue corresponding to ICaBP Pro-20 in both the α and β-subunits could destabilize the N-terminal Ca^{2+} binding fold due to an increase in the flexibility of the loop. Moreover, electrostatic effects due to the presence of a cluster of 5 basic residues in the N-terminal Ca^{2+} binding loop could inhibit the approach of a Ca^{2+} ion as well as deter the approach of a second Ca^{2+} ion to the EF hand site. Hence, a combination of electrostatic effects and conformational differences could account for the lower affinity of the S-100 protein for Ca^{2+} relative to ICaBP. In addition, the N-terminus of both the α and β-subunits of the S-100 protein are characterized by the presence of histidine residues within or near the Ca^{2+} binding loop (His 25β, His 16α and His18α). The protonation of His-25β had been suggested to be responsible for the pH dependence of Ca^{2+} binding to the S-100 protein (Szebenyi *et al.*, 1981; Szebenyi and Moffat, 1986). At physiological pH, the presence of a protonated His-25β (p*K* ~ 7) would further increase the positive charges in the N-terminal Ca^{2+} binding domain which contains a cluster of basic residues and this could inhibit the approach of a Ca^{2+} ion to this region of the molecule. Furthermore, the hydrogen bond(s) and hydrophobic interactions made by the nonprotonated histidine could be disrupted upon lowering the pH from 8.4 to 7.5, and this could lead to a destabilization of the N-terminal Ca^{2+} binding loop. Hence, this has led to the postulation that the single Ca^{2+} bound to each of the α and β subunits of the S-100 protein at pH 7.5, would occupy the C-terminal EF hand site (Szebenyi and Moffat, 1986). Based on the results of our spectroscopic studies which indicated that, at pH 7.5, Ca^{2+} binding to the α-subunit perturbs the single Trp at the C-terminus, it can be inferred that the Ca^{2+} binding site is probably located in this region of the molecule, in close proximity to Trp-90, Tyr-74, Phe-88 and Phe-89, since the latter chromophores are also affected by this metal ion.

The presence of 2 Ca^{2+} binding sites on the α-subunit at pH 8.4 raises the possibility that the higher pH may induce the protein to undergo a gross conformational change resulting in the unmasking of a second Ca^{2+} binding site. However, the observation that the $[\theta]_{222}$ values of the apoprotein did not vary significantly between pH 6.5 and 8.4 (Table V), suggested that raising the pH to 8.4 had no effect on the secondary structural content of the α-subunit. Furthermore, the results from far UV CD (Table IV) and UV difference spectroscopy (Table VIII) indicated that the binding of 1 and 2 Ca^{2+} ions to the α-chain at pH 7.5 and 8.4, respectively, induced effects of similar magnitude at both pH values. The results imply that, at pH 8.4, the binding of one of the two Ca^{2+} ions is responsible for most of the Ca^{2+} induced spectral changes while the other Ca^{2+} exerts a minimal effect. In this respect, our findings are similar to those reported by Baudier *et al.*, (1986). The latter group demonstrated that 4 Ca^{2+} ions bind cooperatively to αα homodimer at pH 8.4 and that the binding of the first two equivalent Ca^{2+}/mole αα accounted for most of the Ca^{2+} induced conformational changes. Moreover, based on the observation that the fluorescence of Trp 90α increased significantly after the binding of the first two Ca^{2+} to S-100αα , it can be reasoned that upon raising the pH to 8.4 , the Ca^{2+} binding site located at the C-terminus of the protein binds Ca^{2+} much tighter ($K_d \sim 10^{-5}$ M) while a second Ca^{2+} binding site which binds Ca^{2+} with a lower affinity (10^{-4} M) became available. The second Ca^{2+} ion is presumably bound at the N-terminus, between residues 19α-32α since Szebenyi *et al.*, (1981) suggested that the deprotonation of His 25β within the Ca^{2+} binding loop might be responsible for the pH dependent increase in this site's affinity for Ca^{2+}. Even though the α-chain lacks a histidine at the position corresponding to His 25β, it is possible that a pH induced change in the local environment of the 2 nearby histidines (His 16α and His 18α) can influence the affinity of the N-terminal pseudo invariant site for Ca^{2+}.

Contrary to the far UV CD and UV absorption difference results, we have observed that the Ca^{2+} induced fluorescence enhancement of the tryptophan in S-100α was pH dependent (Table IX). Furthermore, the shapes of the pH titration curves (Fig. 19) are similar to those reported by Shinitzky and Goldman (1967) in their studies of tryptophan fluorescence quenching by protonated histidine residues in peptides and proteins. In these studies, the p*K* values of the histidines ranged from 5.8 to 7.22. Since we have observed that the Ca^{2+} induced fluorescence enhancement is affected by a chemical group with p*K* of 7.05 and that the N-terminus contains both of the histidine residues in the α-chain (at positions 16 and 18), this raises the possibility that the conformational change induced by Ca^{2+} binding may bring the tryptophan sufficiently close to the protonated histidine residues, resulting in the formation of a charge complex and lead to the quenching of Trp fluorescence at pH 7.5. At pH 8.4, deprotonation of these histidines would abolish such a complex, resulting in an increase in the Trp fluorescence intensity. Hence, the deprotonation of His 16α and His 18α may account for the pH dependence of the Ca^{2+} induced increase in tryptophan fluorescence.

From Table V, it can be seen that titration of the metal free α-subunit from pH 8.4 to 5.6 did not result in any significant alteration in the $[\theta]_{222}$ values. These results are in contrast to those observed for Tn-C (Hincke *et al.*,1978), whereby acid titration of both skeletal and cardiac Tn-C, in the absence of Ca^{2+}, resulted in a 36% and 20% increase in the $[\theta]_{222}$ values, respectively, as the pH was decreased from 7.0 to 5.5. Since Murray and Kay (1972) had previously reported that the saturation of the 4 Ca^{2+} binding sites on skeletal Tn-C resulted in a 60% increase in the $[\theta]_{221}$ value, the results suggest that protons can partially mimic the role of Ca^{2+} in evoking a conformational change. In the case of the α-subunit, the fact that Ca^{2+} only induced a 10% reduction in $[\theta]_{222}$ value may make it difficult to monitor the effect of protonation on S-100, since protons can only mimic the conformational change evoked by Ca^{2+} to a limited extent.

(ii) Zn^{2+} binding properties

In the presence of Zn^{2+}, the α-subunit undergoes a gross conformational change. The fact that Zn^{2+} has no effect on S-100b in the far UV CD region (Mani and Kay, 1983b) suggests that the decrease in the α-helical content observed in S-100a upon the binding of this metal ion is localized in the α-subunit, in agreement with our far UV CD results. On the other hand, the near UV CD studies suggest that Zn^{2+} only perturbed the tyrosine chromophores in the α-subunit to a small extent, compared to the large perturbations in the environment near the tyrosine and phenylalanine residues in S-100a (Mani and Kay, 1983b). Furthermore, the single Trp is not affected to any great extent by Zn^{2+}, as indicated by UV difference and fluorescence spectroscopy. Hence, we were unable to determine the affinity of the α-subunit for this metal ion using the spectroscopic techniques described in this study.

Using far UV CD spectroscopy, we have demonstrated that both Ca^{2+} and Zn^{2+} can bind and induce a conformational change in the presence of each other (Table IV). In addition, the final CD spectra are similar irrespective of the order of addition of metal ions. These results may be taken as evidence to indicate that the α-subunit possesses different Zn^{2+} and Ca^{2+} binding sites, a finding similar to that reported for the other 2 isoforms of S-100 protein (Mani and Kay, 1983b; Baudier and Gerard, 1983; Baudier *et al.*, 1986).

In general, in a number of Zn^{2+} binding proteins, the Zn^{2+} ion is tetrahedrally coordinated. In the case of thermolysin, the coordinating residues are 2 histidines, 1 glutamic acid residue and a water molecule (Colman *et al.*, 1972) while in carbonic anhydrase, 3 histidines and a water molecule serve to coordinate this metal ion (Blundell *et al.*, 1972). In addition, the sulfhydryls of cysteines can also act as ligands as observed in aspartate carbamoyltransferase (Monaco *et al.*, 1978). More recently, in the Zn^{2+} binding domains of a number of nucleic acid binding proteins, each Zn^{2+} ion is coordinated by 2 pairs of invariant cysteines and histidines in a 30 residue loop known as the "Zn^{2+} finger" motif (Klug and Rhodes, 1987). In the case of S-100α, the C-terminal region is characterized by a single cysteine and tryptophan at positions 85 and 90 as well as one tyrosine at position 74. The fact that Zn^{2+} binding to the α-subunit does not result in a significant alteration in the environment near Trp 90α, suggests that the Zn^{2+} binding site is located away from the C-terminal region. On the other hand, the N-terminal region contains both of the histidines found in S-100α (at positions 16 and 18) as well as the remaining tyrosine at position 26. Since we have observed that one or more tyrosine(s) is affected by binding of Zn^{2+} ions, it is likely that Tyr 26 is the one involved in the Zn^{2+} binding to the α-subunit. Hence, the single Zn^{2+} binding site on the α-subunit is probably located in the N-terminal region, in close proximity to the 2 histidines and Tyr 26. The differences in Zn^{2+} binding properties observed between the α-subunit and S-100a (αβ) may be due to a decrease in the number of histidine residues in the α-chain that can coordinate Zn^{2+}. Each of the β-subunits possesses 5 histidines, 2 of which are present in the C-terminus (His 85β, His 90β), in close proximity to a cysteine at position 84. It is possible that the C-terminus of the β-subunit may possess additional Zn^{2+} binding sites in contrast to the α-subunit, which lacks these coordinating residues at the corresponding positions. Baudier *et al.* (1986), using a flow dialysis method to study $^{65}Zn^{2+}$ binding to various S-100 isoforms, reported that S-100b binds 8 mol Zn^{2+}/mol dimer with K_d values of 10^{-6} to 10^{-8} M, while S-100a binds 4 mol Zn^{2+}/mol dimer with a K_d value of 10^{-5} to 10^{-7} M. In the case of the α-subunit, there is an indication that it binds 3 Zn^{2+}/dimer with an affinity of approximately 1×10^{-5} M at pH 7.5. Moreover, the binding of 4 mol Zn^{2+}/mol ββ, presumably at the C-terminus, increases the affinity of the protein for Ca^{2+} by 0.5 and 1 order of magnitude respectively, at pH 7.5 and 8.3 (Baudier and Gerard, 1983; Baudier *et al.*, 1986). In the case of S-100a, no increase in the affinity for Ca^{2+} was observed for the Zn^{2+} bound protein (Baudier and Gerard, 1983). In our study, we have also reported that the presence of 3.55 mol Zn^{2+}/mol α-dimer has no effect on the affinity of the protein for Ca^{2+} (Table X). Hence, these studies suggest that α and β subunits have different Zn^{2+} binding properties and that the Zn^{2+} effect is minimal on the α-subunit of the S-100 protein. The differences in the Zn^{2+} binding properties between S-100 isoforms have led to the use of Zn^{2+} dependent affinity chromatography on a phenyl Sepharose column to separate S-100a from S-100b protein (Baudier *et al.*, 1982a). In the presence of 0.1 mM Zn^{2+}, S-100b was selectively bound to the column and subsequently eluted upon the application of a buffer containing EDTA. On the other hand, S-100a did not bind to the column and was eluted in the void volume.

(iii) Effect of K^+

In the case of K^+, addition of this metal ion to the α-subunit has no apparent effect on the conformation of the protein. However, when K^+ was subsequently added to the

Ca^{2+} saturated α-subunit, it antagonized the Ca^{2+} induced effect as revealed by near UV CD and UV difference spectroscopy. The antagonistic effect is small since K^+ only caused a 10% reduction in the Δε values of the Ca^{2+} induced UV difference spectrum, in agreement with the observation of Masure *et al.* (1984) with their preparation of S-100α. Nevertheless, the affinity of the α-subunit for Ca^{2+} is reduced in the presence of KCl (Table X). Furthermore, at pH 8.4, from the fluorescence titration results, there was an indication of one class of Ca^{2+} binding site with a K_d value of 10^{-4} M, suggesting that K^+ antagonized Ca^{2+} binding to the high affinity site, or alternatively, K^+ caused a decrease in the K_d value of the tight Ca^{2+} binding site. However, the Ca^{2+} binding stoichiometry study performed in the presence of 90 mM KCl at pH 8.4, revealed that 2 Ca^{2+} ions can still bind to each α-subunit under these conditions(Table III). These results suggest that the antagonistic effect of K^+ is probably due a non-specific modulation of the Ca^{2+} binding sites by electrostatic shielding of K^+ ions. Furthermore, both near UV CD and UV difference spectroscopy have shown that in the presence of KCl, Ca^{2+} is capable of inducing a conformational change in S-100α, with magnitude similar to that observed in the absence of KCl (Table VII and VIII). Hence, from these results, it can be concluded that 2 Ca^{2+} binding sites still exist on each α-subunit at pH 8.4 in the presence of KCl. However, K^+ binding is likely to affect the high affinity Ca^{2+} binding site at the C-terminus, presumably by inducing a subtle change in the local environment, thereby causing this site to bind Ca^{2+} less tightly, resulting in a decrease in the K_d value to 10^{-4} M. It is not clear whether the monovalent cation also affects the pseudo invariant Ca^{2+} binding site at the N-terminus since the K_d value obtained from fluorescence titration in the presence of KCl at pH 8.4 represents the average affinity of the 2 Ca^{2+} binding sites that are present on the α-subunit at this pH. In the case of the β-subunit that exists in the heterodimer S-100a and the homodimer S-100b, the affinity for Ca^{2+} is also reduced in the presence of KCl (Mani *et al.*, 1983a; Baudier *et al.*, 1986). Moreover, K^+ was more effective in reversing most of the Ca^{2+} induced spectral changes in S-100a and S-100b than in the α homodimer (Mani *et* *al*., 1983; Mani and Kay, 1983a). It is possible that the 2 α-chains in the homodimer may exhibit subtle folding arrangements that are different from the β-subunit, which leads to a modulation in the antagonistic effect of the monovalent cation on the Ca^{2+} binding sites located on each of the subunits. The antagonistic effect of K^+ towards Ca^{2+} binding is not unique to S-100 protein. Haiech *et al.* (1981) and Keller *et al.* (1982) also reported that the affinity of the 4 Ca^{2+} binding sites on calmodulin was reduced in the presence of KCl. On the other hand, the presence of 150 mM KCl had no effect on the conformation of the protein (Martin and Bayley, 1986).

(iv) Effect of Mg^{2+}

Addition of Mg^{2+} to S-100α induces no apparent effect on this isoform of S-100 protein. Moreover, in the presence of Mg^{2+}, Ca^{2+} was able to induce a conformational change in the α-subunit and the apparent K_d value for Ca^{2+} remained unaffected by the presence of this metal ion. Taken together, these results suggest that the Ca^{2+} binding site is specific for this metal ion, in agreement with that observed for S-100b (Mani and Kay, 1983; Matsuda, 1988).

(v) Tb^{3+} *binding properties*

The Ca^{2+} analog, terbium, was used to probe the Ca^{2+} binding site on the α-subunit of S-100a protein. Terbium binding assays at pH 6 5, revealed that 1 Tb^{3+} is bound per α-chain which contained one EF hand type Ca^{2+} binding site (Isobe and Okuyama, 1981a). Tb^{3+} binding to the α-subunit resulted in a 4% reduction in the α-helical content similar to that observed with Ca^{2+}. On the other hand, the results from the near UV CD and UV difference spectral studies suggested that Tb^{3+} exerted a small effect on the microenvironment of the α-subunit. Nevertheless, the effect was significant, since Ca^{2+} at a similar metal : protein ratio (0.5:1) failed to induce any UV difference spectrum. The larger effect of terbium may well be due to the fact that Tb^{3+} possesses a larger charge to volume ratio compared to Ca^{2+}, thereby favouring higher ligand coordination and resulting in tighter binding. The enhanced luminescence properties of Tb^{3+} when bound to the α-chain are due to radiationless energy transfer from a nearby aromatic residue donor (Dockter, 1982; Horrocks, 1982). In the case of the α-subunit which contains 1 Trp and 2 Tyr, the resolution of the identity of the energy donor remains unsolved, since sensitized Tb^{3+} emission was observed upon excitation of either the tryptophan or the tyrosines. Moreover, Tb^{3+} binding to S-100α did not affect the intrinsic fluorescence to any great extent, suggesting that the metal ion binding site on the protein may not be sufficiently close to the aromatic donor chromophore to allow energy transfer to occur efficiently. In the case of other calcium binding proteins such as calmodulin, which binds 4 Tb^{3+}/mol protein, the distance between the energy transfer pair (tyrosine and Tb^{3+}) lies within 5 Å (Wallace *et al.*, 1982).

An earlier study on the Tb^{3+} binding properties of S-100a reported that this isoform of S-100 binds 2 mol Tb^{3+}/ mol protein (Mani and Kay, 1987b). Since we have established that 1 Tb^{3+} is bound per α-chain, this suggests that the β-subunit in the heterodimer also possesses 1 Tb^{3+} binding site. Despite the fact that both isoforms underwent a conformational change in the presence of this metal ion, the perturbation of the environment near the aromatic chromophore in the α-subunit was small compared to that observed in S-100a. The differences in Tb^{3+} binding properties may reflect subtle changes in the local environment near the metal ion binding site between an α and β chain in S-100a and the α-subunit which exists as a homodimer.

(vi) Stability of the α-subunit

The thermal unfolding study of the metal free α-subunit indicated that the protein is relatively stable to denaturation. In this respect, the S-100 protein is similar to ICaBP in which thermal unfolding of the apoprotein is only half completed at 90 °C (Wendt *et al.*, 1988), compared to a transition midpoint for apo-calmodulin at 55 °C (Brzeska *et al.*, 1983). On the other hand, when GdnHCl was used as a denaturant, extensive unfolding was observed which resulted in a 95% reduction in the $[\theta]_{221}$ value compared to 35% observed in the thermal denaturation study. Furthermore, the binding of Ca^{2+} to the α-subunit enhances its stability, since a higher concentration of GdnHCl was required to attain the mid-point of transition between the 2 conformational states, compared to the metal free protein. In the case of cardiac and skeletal Tn-C, Ca^{2+} binding to these proteins also enhances their stability against thermal denaturation, resulting in a 30% reduction of initial ellipticity values compared to 60% in the case of the metal free protein (McCubbin *et al.*, 1980).

(vii) Molecular weight studies of the α-subunit

The sedimentation equilibrium studies performed with S-100α have established a weight average molecular weight of 11,020 ± 1000 and 21,370 ± 1000 for this subunit in the presence and absence of denaturants, respectively. This suggests that the α-subunit, isolated from S-100a (αβ) in the presence of 8 M urea, can reassociate to form a homodimer with an apparent molecular weight of 21,000 under native solvent conditions. Moreover, the experiments performed in 6 M GdnHCl in the presence and absence of reducing agent, gave virtually the same results, suggesting that the 2 polypeptide chains are not held together by covalent forces. These results also imply that the 2 structurally related α or β subunits of S-100 protein can combine to yield various isoforms comprised of homodimers (αα and ββ) as well as heterodimers (αβ). The apparent molecular weight obtained for the α-subunit in the presence of 1 mM Ca^{2+} and 0.2 mM Zn^{2+} suggests both of these metal ions induce the protein to undergo aggregation to a limited extent.

In summary, with the isolation of the α-subunit using the preparative methodology reported in the previous chapter, we were able to examine its physico-chemical and metal ion binding properties. Since our preparation of the α-subunit exists as a homodimer under native solvent conditions and has an amino acid composition similar to that reported for the

S-100a$_0$ isoform (Isobe *et al.*, 1983), it is probable that the 2 proteins are identical. Using a variety of spectroscopic techniques, we have demonstrated that Ca^{2+} and Zn^{2+} induce different conformational changes upon binding to the α-subunit. These studies have shown that while the overall effect of Ca^{2+} on S-100αα is similar to that observed in S-100a (αβ), the Zn^{2+} effect is different, suggesting that in spite of the extensive sequence homology between the α and β subunits, they have different metal ion binding properties. It is conceivable that these differences may account for different physiological roles for the α and β subunits and may well be the rationale for the existence of the various isoforms of S-100 protein in the brain.

V. FLUORESCENCE PROBE STUDIES ON THE CALCIUM AND ZINC BINDING PROPERTIES OF THE α-SUBUNIT OF BOVINE BRAIN S-100a PROTEIN.[1]

Fluorescence spectroscopy has been widely used to study protein conformation. The intrinsic fluorescence of proteins, which arises mainly from tryptophan and tyrosine, is highly dependent on their environment. Hence, this provides an effective tool to study protein ligand interactions. However, it is also possible to study protein conformation using extrinsic fluorescence probes which can be introduced into the protein either by chemical coupling or simple binding. This technique is particularly useful for proteins that lack intrinsic fluorophores. In this chapter, we have chosen one example from each of the two classes of fluorescence probes to study the effect of metal ion ligand binding to the α-subunit and the S-100a protein at pH 7.5. In the first example, the fluorescence probe, 2-p-toluidinylnaphthalene-6-sulfonate (TNS), which binds to hydrophobic surfaces on proteins in a non-covalent manner, was employed (McClure and Edelman, 1966). In the second example, 6-Acryloyl-2-dimethylaminonaphthalene (Acrylodan), which interacts specifically with thiol groups (Prendergast *et al.*, 1983), was covalently linked to the α-subunit and S-100a protein. These probes are generally non fluorescent by themselves, but become highly fluorescent when bound to proteins. Moreover, the observed fluorescence intensity and their wavelength of emission maximum are generally a function of the polarity of the medium in which the probe is situated. Hence, one can use these probes as reporter groups to study the effect of metal ion binding on the local environment of the α-subunit and to compare its binding with that observed with its parent molecule, S-100 protein.

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[1] A version of this chapter has been published: Leung, I.K.M., Mani, R.S. and Kay, C.M. (1987) *FEBS Lett.* 214, 35-40.

A. Interaction of TNS with the α-subunit and S-100a protein

1. Effect of metal ion binding to S-100α and S-100a on TNS Fluorescence. It has been postulated that the binding of Ca^{2+} to a mixture of S-100 protein induces a conformational change involving the partial exposure of a hydrophobic domain (Calissano *et al.*, 1974). In this study, the hydrophobic probe, TNS, was used to examine the effect of Ca^{2+} binding to the α-subunit and to its parent molecule, S-100a protein. In polar solvents, TNS has negligible fluorescent properties, but becomes highly fluorescent in apolar media or after non-covalent interaction with hydrophobic surfaces on proteins (McClure and Edelman, 1966). In the absence of the S-100 protein, the TNS exhibited very low fluorescence in 0.1 M Tris/HCl, pH 7.5, and was characterized by a broad emission maximum at approximately 480 nm (data not shown) As seen in Figs. 30a and 30b, the fluorescence of TNS of the metal free α-subunit and S-100a protein (at a probe : protein molar ratio of 3:1), was negligible. Addition of 1 mM Ca^{2+} to the α-subunit and S-100a protein produced a 9 and 7 fold enhancement in TNS fluorescence, respectively, accompanied by a blue shift in the emission maximum in both proteins to 445 ± 1 nm, implying that the probe occupied a more hydrophobic environment. On the other hand, addition of 0.4 mM Zn^{2+} induced a large blue shift, with the emission maximum occurring at 430 ± 1 nm for both the α-subunit and S-100a protein. However, with the former, the TNS fluorescence did not change significantly, whereas a 14 fold enhancement was observed for the latter.

2. Affinity of the α-subunit and S-100a protein for TNS. The affinity of the α-subunit and S-100a protein for the TNS probe in the presence of Ca^{2+} was estimated by monitoring the increase in the probe's fluorescence as it was added to the protein. From the slope term of a double reciprocal plot of TNS fluorescence and its concentration (Fig. 31), an apparent K_d value of $4.8 \pm 0.1 \times 10^{-5}$ M was obtained for the α-subunit. A similar analysis of the titration data for S-100a protein (titration data not shown) gave a K_d value of $6.5 \pm 0.1 \times 10^{-5}$ M.

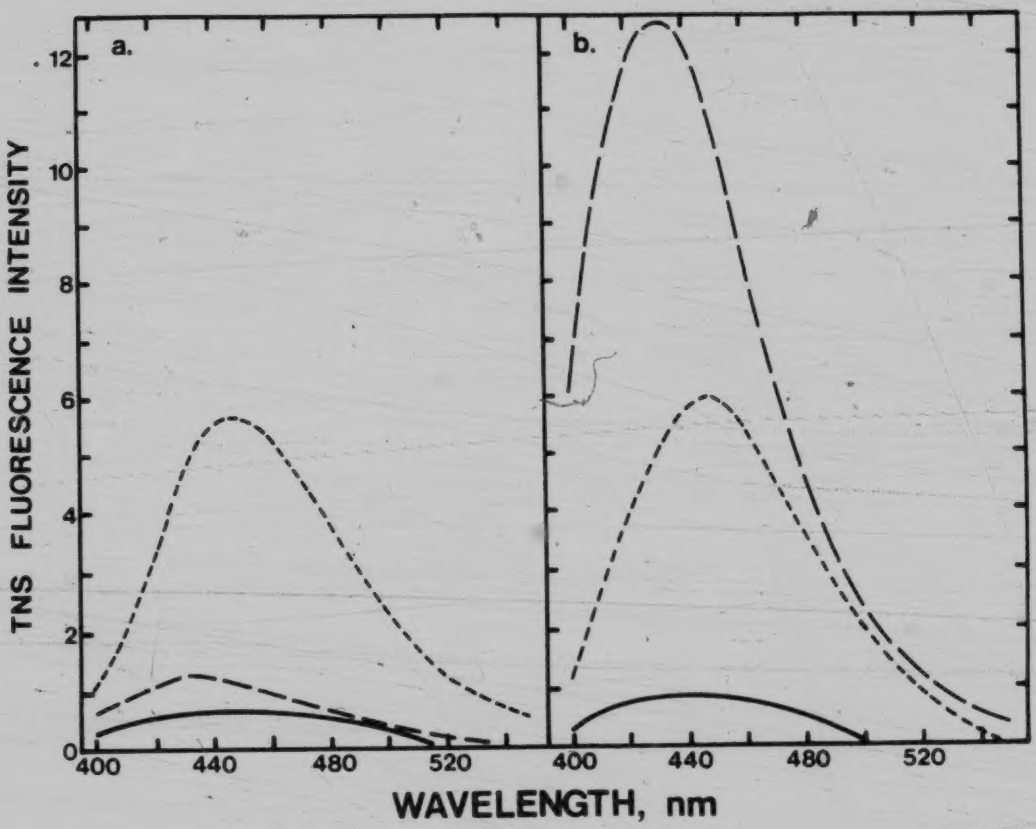

Fig. 30. Fluorescence emission spectra of the TNS bound to (a) α-subunit at a concentration of 5.3 μM, in 0.1 M Tris/HCl, pH 7.5 with 15.1 μM TNS and, (b) S-100a protein at a concentration of 5.3 μM, in 0.1 M Tris/HCl, pH 7.5 with 15.1 μM TNS, under varying conditions; (——) in the absence of any divalent cations; (------) in the presence of 1 mM Ca^{2+} and (— —) in the presence of 0.4 mM Zn^{2+}. The excitation wavelength was set at 345 nm. Copyright (1987) Federation of European Biochemical Society.

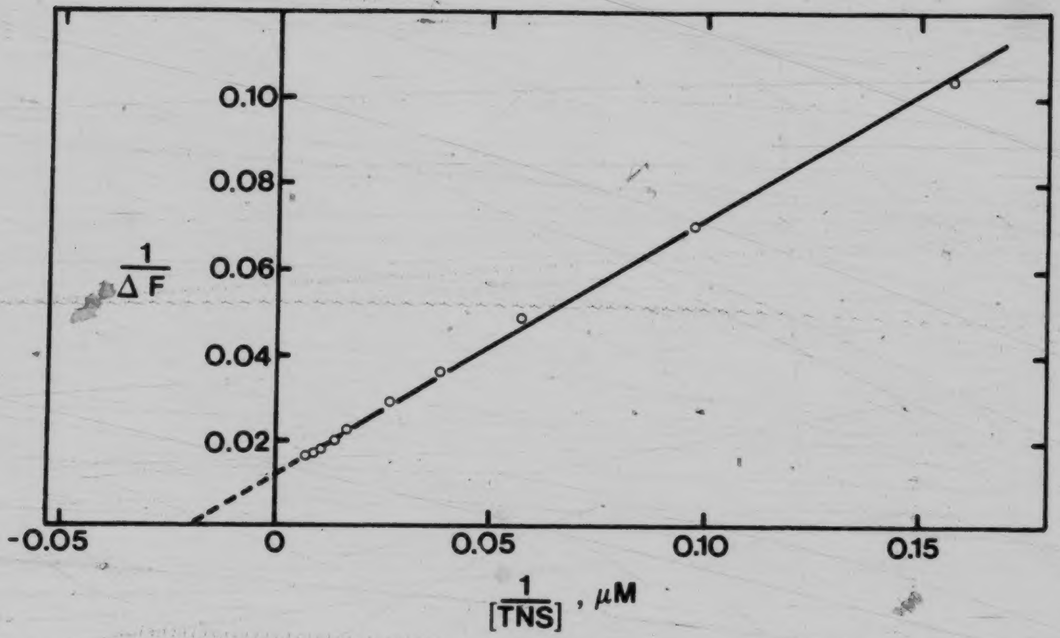

Fig. 31. Affinity of the α-subunit for TNS in the presence of Ca^{2+}. A double reciprocal plot of TNS fluorescence (at 445 nm) and its concentration are given. The protein at a concentration of 9.9 μM, was in 0.1 M Tris/HCl, pH 7.5 and 1 mM Ca^{2+}. The excitation wavelength was set at 345 nm.

B. Interaction of Acrylodan with the α-subunit and S-100a protein.

In the previous chapter, using a variety of spectroscopic techniques, we have demonstrated that Ca^{2+} binding to the α-subunit perturbed the single tryptophan located at the C-terminal region of the molecule. In addition to the single tryptophan at position 90, the α-subunit is also characterized by a single cysteine at position 85. The cysteines in the S-100 protein are of considerable interest since it had been suggested that the state of the sulfhydryl groups contributed to their electrophoretic heterogeneity (Calissano *et al.*, 1969), conformational state (Rusca and Calissano, 1970) and reactivity towards specific antibodies (Dannies and Levine, 1971). In order to examine the nature of the change in the environment of the sulfhydryl group upon Ca^{2+} and Zn^{2+} binding to the α-subunit, we have employed the thiol specific, polarity sensitive fluorescent probe, Acrylodan, to label the protein. The α-subunit is an ideal system for this purpose, since it contains only 1 cysteine at position 85.

1. Reaction of the α-subunit and S-100a with DTNB.

Before labelling the α-subunit with Acrylodan, the reactivity of the single cysteine at position 85 was examined by titrating with the thiol specific reagent, DTNB (Ellman, 1959), at a reagent : sulfhydryl ratio of 10:1. As seen in Fig. 32, after 1 hour, 0.1 and 0.95 SH/chain was titrated in the presence of 1 mM EDTA and 6 M GdnHCl, respectively. The results suggested that the sulfhydryl was essentially inaccessible in the apoprotein, but became exposed upon denaturation. In the presence of 0.4 mM Zn^{2+}, the reaction with DTNB was slow, and after 1 hour, 0.2 thiol / chain reacted whereas in the presence of 1 mM Ca^{2+}, 0.65 SH group was able to interact with DTNB. Hence the sulfhydryl group was more exposed to the solvent in the presence of Ca^{2+}.

The reactivity of the cysteines in S-100a protein at position 85 on the α-chain and at positions 68 and 84 on the β-chain were also investigated. As seen in Fig. 33, in the absence of metal ions, approximately one of the three sulfhydryls reacted with DTNB as opposed to 2.8 thiols/mole protein being titrated upon unfolding the S-100a protein in 6 M

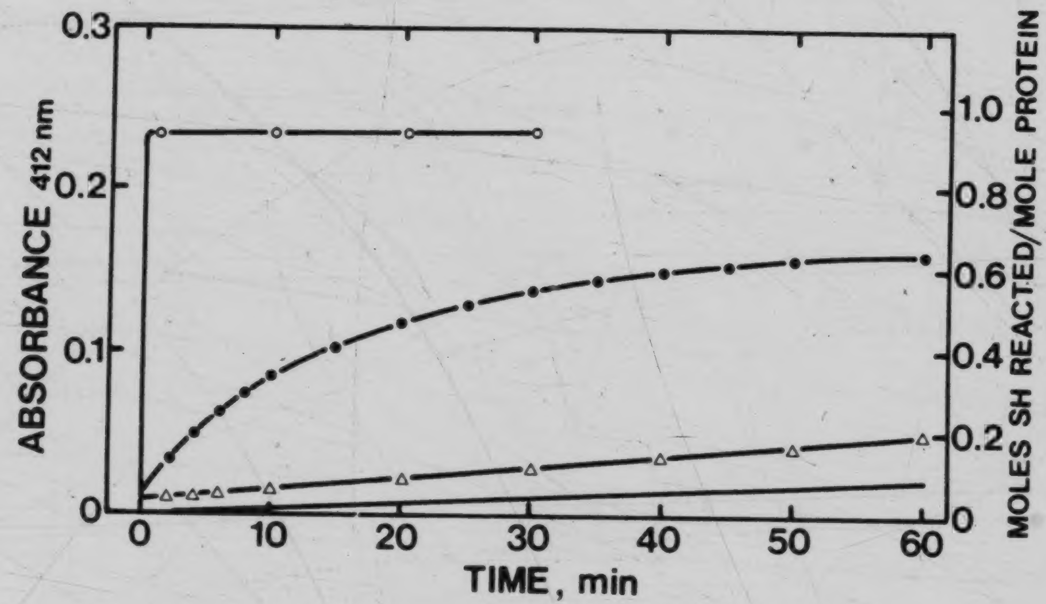

Fig. 32. Reactivity of the sulfhydryl group of the α-subunit of S-100a protein towards DTNB. The α-subunit (18 μM) was dissolved in 50 mM Mops, pH 7.0 and reacted with DTNB under varying conditions; (——) in the presence of 1 mM EDTA; (— △ —) in the presence of 0.4 mM Zn^{2+}; (— ● —) in the presence of 1 mM Ca^{2+} and, (—○—) unfolded in 6 M GdnHCl. The rate of the reaction was monitored by following the absorbance at 412 nm.

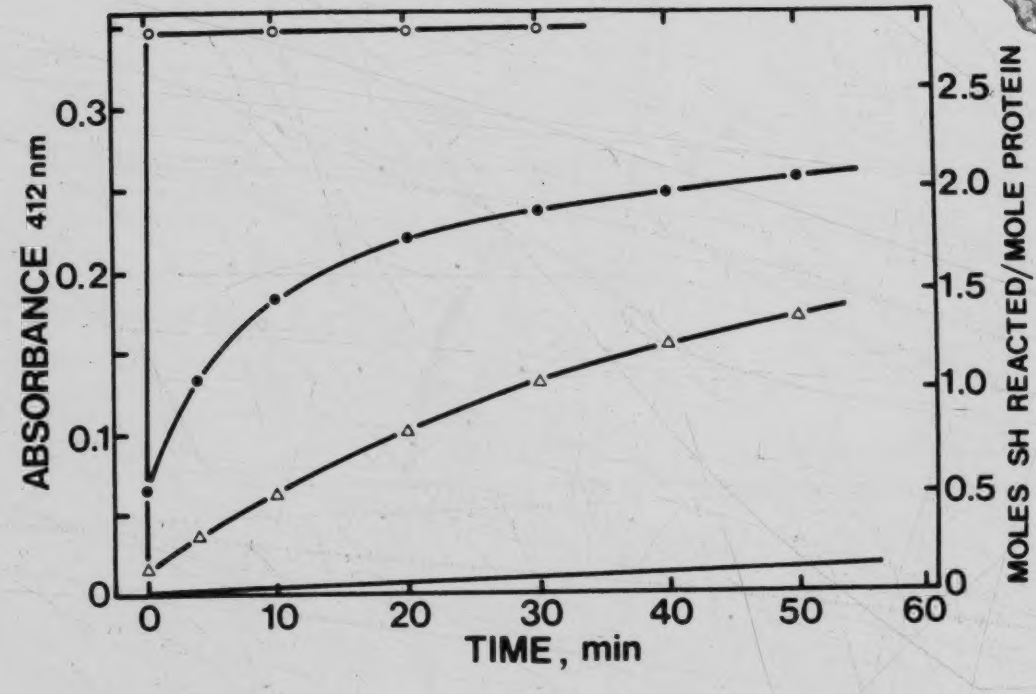

Fig. 33. Reactivity of the thiol groups of S-100a protein towards DTNB. The S-100a protein (18 μM) was dissolved in 50 mM Mops, pH 7.0 and reacted with DTNB under varying conditions; (——) in the presence of 1 mM EDTA; (—Δ—) in the presence of 0.4 mM Zn^{2+}; (— ● —) in the presence of 1 mM Ca^{2+} and, (— ○ —) unfolded in 6 M GdnHCl. The rate of the reaction was monitored by following the absorbance at 412 nm.

GdnHCl. The slightly lower value (i.e., less than 3.0) could be due to autooxidation of the thionitrophenyloate anion under denaturation conditions (Butterworth *et al.*, 1967). Under native solvent conditions, in the presence of Ca^{2+} and Zn^{2+}, 2.1 and 1.4 SH/mole protein were able to interact with DTNB, respectively.

2. Labelling of S-100α and S-100a protein with Acrylodan.

The labelling of the α-subunit with the thiol specific apolar fluorescent probe, Acrylodan, was performed in the presence of 1 mM Ca^{2+} since the DTNB study suggested that cysteine 85α was more reactive in the presence of this metal ion. Under these conditions, 0.31 thiol groups/ α-chain reacted with the probe. In the case of S-100a protein, based on the results obtained from the DTNB study reported above as well as those of Mani and Kay (1985a), one of the three sulfhydryls in the apoprotein was more reactive compared to two thiols titrated in the presence of Ca^{2+}. Therefore, in order to maintain a fluorophore : protein labelling ratio of less than 1 as observed with the α-subunit, S-100a protein was reacted with Acrylodan in the presence of 1 mM EDTA. The number of sulfhydryl groups labelled was 0.35.

The fluorescence spectra of the Acrylodan labelled α-subunit are shown in Fig.34. When the apoprotein was excited at 380 nm, the wavelength of the emission maximum occurred at 484 ± 2 nm. In the presence of Ca^{2+}, the emission maximum of the protein underwent a red shift to 504 ± 2 nm with no significant increase in the fluorescence intensity. When the protein-Acrylodan adduct was dissolved in 6 M GdnHCl, the fluorescence λ_{max} shifted to 525 ± 2 nm. This implies that the fluorophore, when fully exposed to the solvent, has a λ_{max} around 525 ± 2 nm. Hence, it would appear that the 6-acryl-2-dimethylaminonaphthalene moiety attached to the single thiol group in the apoprotein is in a fairly hydrophobic environment. The red shift observed upon Ca^{2+} addition to the α-subunit is due to a conformational change induced by metal ion binding, resulting in the probe moving to a more polar microenvironment. Addition of Zn^{2+} to the

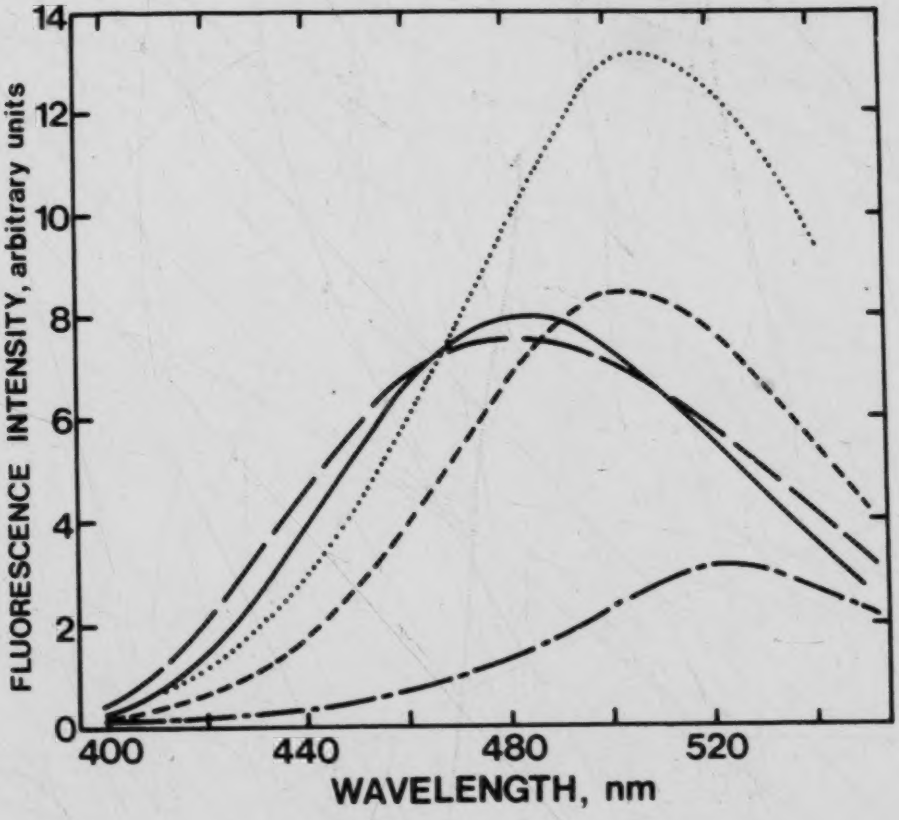

Fig. 34. Fluorescence emission spectra of the Acrylodan labelled α-subunit of S-100a protein in 0.1 M Tris/HCl, pH 7.5 at 25°C under varying conditions: (——) in the absence of divalent cations; (------) in the presence of 1 mM Ca^{2+}; (— —) in the presence of 0.4 mM Zn^{2+}; (••••) in the presence of 1 mM Ca^{2+} and 0.4 mM Zn^{2+} and, (— - —) unfolded in 6 M GdnHCl. The concentration of the protein was 1.2 μM and an excitation wavelength of 380 nm was used. Copyright (1987) Federation of European Biochemical Society.

labelled protein had no significant effect on either the wavelength of the emission maximum (481 ± 2 nm) or the fluorescence intensity, suggesting that Zn^{2+} was not altering to any extent the conformation around the single thiol group and for this reason the labelled protein was titrated only with calcium. The titration curve generated by monitoring the maximum change in fluorescence intensity (at 450 nm), as a function of Ca^{2+} concentration is shown in Fig. 35, from which a K_d value of $1.2 \pm 0.1 \times 10^{-5}$ M was derived and this corresponded to the Ca^{2+} concentration required to induce a 50% change in the observed fluorescence intensity. In the presence of 90 mM KCl, the protein binds Ca^{2+} with lower affinity (K_d, 3.31×10^{-5} M) implying that KCl acts as an antagonist toward Ca^{2+}, in agreement with our earlier finding on the native protein. However, if Zn^{2+} was added to the α-subunit after the addition of Ca^{2+} (Fig. 34), it was able to produce a further increase (50%) in the fluorescence intensity at the emission maximum of the Ca^{2+} bound protein (i.e. at 504 nm). Although it had been shown earlier that addition of Zn^{2+} alone did not alter the environment around the labelled thiol, the fact that Zn^{2+} was able to induce a change in the presence of Ca^{2+}, suggested that it could only perturb the labelled thiol after Ca^{2+} had induced a conformational change. On the other hand, Ca^{2+} was able to induce a conformational change in the labelled protein irrespective of whether Zn^{2+} was present or not. It is obvious from these two experiments that Ca^{2+} and Zn^{2+} can bind and perturb the thiol group in the presence of each other and is consistent with our findings with the β-subunit (Mani and Kay, 1986b).

The fluorescence spectra of the Acrylodan labelled S-100a protein are shown in Fig. 36. Addition of Zn^{2+} to the labelled protein caused the emission maximum to undergo a blue shift, from 480 ± 2 nm to 470 ± 2 nm, accompanied by a 20% increase in the fluorescence intensity at the λ_{max}. In the presence of Ca^{2+} or 6 M GdnHCl, the emission maximum occurred at 491 ± 2 nm and 525 nm, respectively. The observed blue shift suggests that Zn^{2+} induced the fluorophore to move to a less polar environment whereas the effect of Ca^{2+} was opposite. The results clearly reveal that the probe occupies

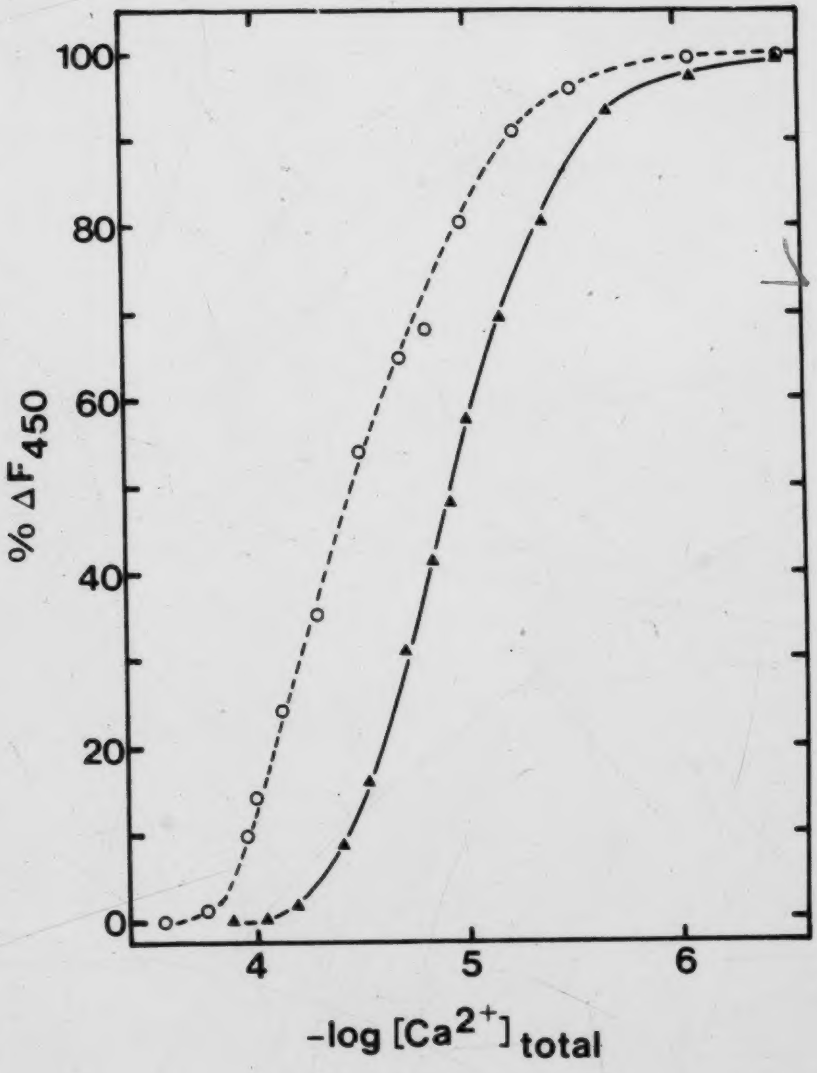

Fig. 35. Fluorescence titration of the Acrylodan labelled α-subunit of S-100a protein at pH 7.5. The percentage change in fluorescence intensity is plotted as a function of the negative logarithm of total Ca^{2+} concentration added to the protein (12 μM), in 0.1 M Tris/HCl, pH 7.5 at 25°C, in the absence of (——), and in the presence of (-----) 90 mM KCl. The protein was excited at 380 nm and the emission monitored at 450 nm.

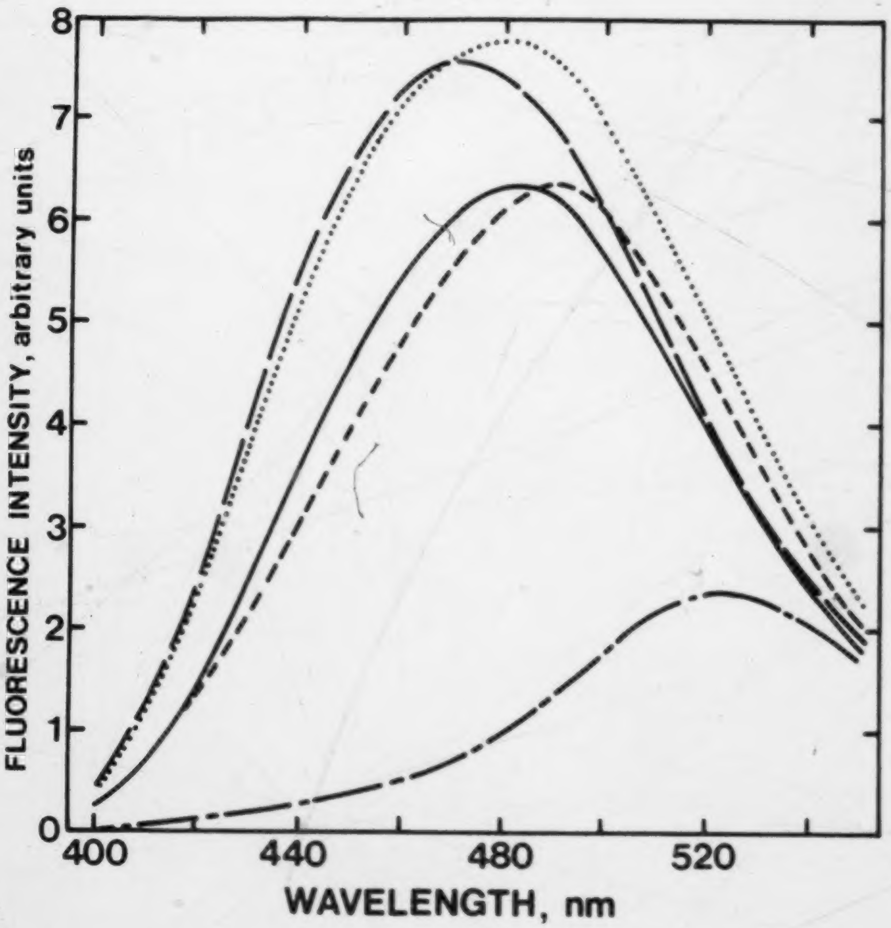

Fig. 36. Fluorescence emission spectra of the Acrylodan labelled S-100a protein in 0.1 M Tris/HCl, pH 7.5 at 25°C under varying conditions: (——) in the absence of divalent cations; (-----) in the presence of 1 mM Ca^{2+}; (— —) in the presence of 0.4 mM Zn^{2+}; (••••) in the presence of 1 mM Ca^{2+} and 0.4 mM Zn^{2+} ,and (— - —) unfolded in 6 M GdnHCl. The concentration of the protein was 1.2 µM and an excitation wavelength of 380 nm was used. Copyright (1987) Federation of European Biochemical Society.

different environments depending on the nature of metal ion bound. Upon comparing the metal ion binding properties of the Acrylodan labelled S-100 protein, it can be seen that Ca^{2+}exerted a similar effect in the region around the labelled thiol in both the α-subunit and S-100a. In contrast, Zn^{2+} was observed to perturb the labelled thiol in S-100a protein only but not in the α-subunit, suggesting that the β-subunit in the αβ heterodimer may possess additional Zn^{2+} binding sites in close proximity to the bound fluorophore.

Far UV CD studies were performed with the α-subunit and S-100a protein that had been labelled with Acrylodan. At pH 7.5, in the absence of metal ions, the $[\theta]_{222}$ value of the labelled α-subunit was -16475 ± 300 $deg.cm^2.dmol^{-1}$, which is similar to that reported previously for the native protein (-16800 ± 300 $deg.cm^2.dmol^{-1}$). Addition of 1 mM Ca^{2+} to the apoprotein resulted in a 6% decrease in the $[\theta]_{222}$ value (from -16475 to -15337 ± 300 $deg.cm^2.dmol^{-1}$). Subsequent addition of 0.4 mM Zn^{2+} to Ca^{2+} bound labelled α-subunit caused the $[\theta]_{222}$ to decrease by another 4%. In the case of the S-100a protein, addition of Ca^{2+} to metal free protein labelled with Acrylodan resulted in a 7% decrease in $[\theta]_{222}$ value (from -17900 to -16700 ± 300 deg. cm^2 $dmol^{-1}$) and this was further decreased by another 4% upon the subsequent addition of Zn^{2+} to the same sample. Taken together, the far UV CD studies suggested that incorporation of the fluorescent probe into the α-subunit and S-100a protein did not result in any significant alteration in the secondary structure of the protein. Moreover, the labelled S-100 protein was able to undergo a conformational change upon the addition of Ca^{2+} and Zn^{2+}, similar to that observed in the native α-subunit as reported in the previous chapter.

C. Discussion

In this chapter, we have employed 2 types of fluorescent probes to study the effect of metal ion binding on the conformation of the α-subunit and S-100a protein. One of the probes, TNS, binds noncovalently to hydrophobic surfaces on the protein (McClure and Edelman, 1966), while the other, Acrylodan, selectively labels thiol moieties in a covalent manner (Prendergast *et al.*, 1983). Moreover, the emission spectra and the quantum yields of the Acrylodan derivatives are highly sensitive to dipolar influences in the environment; hence it can be used as a reporter probe to study conformational changes in the region near the labelled cysteine.

(i) *Interaction of TNS with the α-subunit and S-100a protein*

Fluorescence studies performed with TNS labelled α-subunit and S-100a protein have shown that in the absence of metal ions, the fluorescence of TNS was negligible. On the other hand addition of Ca^{2+} to the labelled α-subunit and S-100a protein in the presence of TNS resulted in a blue shift in the emission spectrum and a large increase in the fluorescence intensity. Since TNS only fluoresces strongly in organic solvents or when bound to certain native proteins, but not in water (McClure and Edelman, 1966), the results suggest that the α-subunit and S-100a protein upon binding Ca^{2+}, underwent a conformational change resulting in the probe occupying a hydrophobic surface on the protein. Using the same probe, Calissano *et al.*,(1974), had also reported a similar finding and demonstrated that 1 mole of TNS was bound / mole Ca^{2+} saturated S-100 protein. The TNS probe could bind either at the N-terminal hydrophobic region (residues 4 - 14) or at the C-terminal end involving residues 74α - 90α (or residues 73-80 in the case of the β-chain) based on the amino acid sequence of the α- and β-subunits (Isobe and Okuyama, 1981a). In the case of the α-subunit, which is characterized by a single tryptophan at position 90, our UV difference spectral data and intrinsic fluorescence measurements reported earlier, have shown that Ca^{2+} binding resulted in the tryptophan becoming more exposed to solvent. Since the tryptophan residue is located on the same hydrophobic sequence (residues 74α - 90α), it is likely that Ca^{2+} binding to the α-subunit induced a conformational change in the C-terminal region near the Trp, resulting in the unmasking of a hydrophobic domain to which the TNS probe can bind. In the case of the other 2 isoforms of S-100 protein, Baudier and Gerard (1983) reported that addition of Ca^{2+} to the TNS labelled S-100b protein, which is composed of 2 β-subunits, resulted in no significant change in TNS fluorescence or the emission maximum, suggesting that the hydrophobic domains on S-100b (residues 4 - 14 and 73 - 80) were not perturbed to any great extent upon Ca^{2+} binding. On the other hand, addition of Ca^{2+} to TNS labelled S-100a protein ($\alpha\beta$ heterodimer) resulted in a blue shift in the emission maximum which was accompanied by an increase in TNS fluorescence. Since Ca^{2+} binding to S-100a protein also induced Trp 90α to move to a more polar environment (Mani and Kay, 1983a), it can be reasoned that the TNS binding site on the Ca^{2+} bound S-100a protein is located in the the C-terminal hydrophobic region of the α-chain, in close proximity to the Trp at position 90.

Although Zn^{2+} binding to TNS labelled α-subunit and S-100a protein resulted in a blue shift in the emission maximum, no significant change in TNS fluorescence was observed with the former, in comparison with a 14 fold enhancement observed in the case of S-100a. Histidine residues are known to act as ligands for coordinating Zn^{2+} (Holmes and Matthews, 1981) and in the α-subunit, they are only found at positions 16 and 18, at the N-terminal region of the protein. Since the addition of Zn^{2+} to the labelled protein did not result in a significant increase in the fluorescence intensity, the result suggests that the probe is located at the C-terminal hydrophobic domain, well away from the histidine residues. In addition to His-15β and His-25β, the β-subunit also possesses additional histidines at position 42 and at the C-terminus, at positions 85 and 90. Hence, in the case of Zn^{2+} bound S-100a protein, due to the presence of additional histidines at the C-terminus of the β-subunit, TNS could bind to one or both of the hydrophobic domains located at each end of the β-subunit, as well as to the C-terminal region of the α-chain, resulting in the observed increase in fluorescence intensity.

Based on the fluorescence titration results, the affinities of the α-subunit and S-100a protein for TNS in the presence of Ca^{2+} ($K_d \sim 5 \times 10^{-5}$ M) are similar to the apparent K_d values of 44 and 77 μM reported for calmodulin and Tn-C, respectively (Tanaka and Hidaka, 1981). Hence, Ca^{2+} binding to S-100 protein also resulted in the exposure of a hydrophobic domain similar to that observed for Tn-C and calmodulin. The observed changes in the hydrophobic properties induced by Ca^{2+} binding have led to the use of Ca^{2+} dependent affinity chromatography on phenyl Sepharose in the purification of S-100 protein from bovine and rat brains (Moore and Joy, 1988). Furthermore, the Ca^{2+} induced changes in the hydrophobic properties of S-100 protein may be physiologically significant since Ca^{2+} bound S-100 protein has been reported to interact with liposomes resulting in an increase in the permeability of liposomal membranes to certain cations (Calissano *et al.*, 1974). The latter group suggested that the Ca^{2+} induced exposure of nonpolar residues in S-100 protein may be important in the formation of a specific hydrophobic site which would allow the Ca^{2+}-S-100 complex to interact with the negatively charged lipid barrier.

(ii) Interaction of Acrylodan with the α-subunit and S-100a protein.

Based on our earlier finding that Ca^{2+} binding to the α-subunit induced a conformational change affecting the C-terminal region near Trp 90, we have chosen the thiol specific fluorescent ligand, Acrylodan, to serve as a reporter probe for this region of the molecule. Under native solvent conditions, the single cysteine at position 85 on the α-subunit reacted in a covalent manner with the 6-acyl-2-dimethylaminonaphthalene moiety. Upon the addition of Ca^{2+} to the labelled protein, the α-subunit underwent a conformational change resulting in the extrusion of the fluorophore into the solvent. Since the emission maxima of Acrylodan derivatives are sensitive to dipolar perturbations in their environment (Prendergast *et al.*,1983), the observed red shift in the emission maximum upon unfolding the protein in 6 M GdnHCl, implied that the probe attached to Cys 85α in the native apoprotein occupied a fairly hydrophobic environment. Fluorescence titrations performed with the Acrylodan labelled α-subunit at pH 7.5, suggested that the labelled protein binds Ca^{2+} with higher affinity (K_d ~ 10^{-5} M) compared to the native protein (K_d ~ 10^{-4} M), though the factor(s) which contributes to the phenomenon is not known. In the previous chapter, the near UV CD studies indicated that Ca^{2+} binding to the α-subunit also perturbed one or more tyrosine and phenylalanine residues. Since the C-terminus of the molecule contains a cluster of these residues, it can be inferred from this study that the Ca^{2+} effect is localized in the region where Tyr-74, Cys-85, Phe-88,-89 and Trp-90 are located. In contrast, Zn^{2+} only induced a small perturbation of the fluorophore, implying that its binding site is not in close proximity to cysteine 85. The small perturbation observed with Zn^{2+} may be attributed to the fact that histidine residues which act as ligands for Zn^{2+} (Holmes and Matthews, 1981), are found at position 16 and 18, well away from cysteine 85 located at the C-terminal end.

In the case of S-100a protein, of the 3 sulfhydryls present in this αβ heterodimer, 2 are located at positions 68 and 84 on the β-chain and the third sulfhydryl is found at 85 on the α-subunit. According to the results from an N-ethyl-2-3,-(^{14}C) maleimide labelling study of S-100 protein (Nika *et al.*, 1982), the reactive thiol occurred at the C-terminal portion of the β-chain at position 84, since HPLC analysis of pepsin cleaved protein revealed this thiol to contain most of the label. Moreover, the other thiol in the β-chain at position 68, presumably in the putative Ca^{2+} binding site as predicted by Isobe and Okuyama (1978), was shielded in the intact protein even in the presence of Ca^{2+}. The single thiol group in the α-chain (Cys-85α) is labelled only when the protein is denatured, suggesting that this thiol is also not readily available for labelling. Hence, the single thiol in S-100a protein that reacted with the fluorescence ligand under benign solvent conditions, probably corresponds to Cys-84 on the β-chain. Upon comparing the metal ion binding properties of the α-subunit and S-100a protein, it can be seen that Ca^{2+} exerts a similar effect on the microenvironment near the probe in both proteins, namely the exposure of the fluorophore to solvent. In contradistinction, the two proteins do not respond to Zn^{2+} binding in a similar manner. In the case of S-100a, probable Zn^{2+} binding to additional histidines at the C-terminus of the β-chain could explain the observed larger effect on S-100a protein compared to the α-subunit.

In conclusion, using the thiol specific probe, Acrylodan, we have demonstrated that Ca^{2+} induced a conformational change in the C-terminal region of the α-subunit and S-100a protein. Moreover, based on the results obtained from the Acrylodan probe, it can be inferred that the hydrophobic domain present at the C-terminus of the α-subunit is unmasked upon the formation of a Ca^{2+}-S-100α complex. In contrast to S-100a protein, Zn^{2+} induced a small perturbation in the α-subunit, suggesting that its binding site is not in close proximity to the labelled cysteine at the C-terminus. The fact that Ca^{2+} and Zn^{2+} can perturb the labelled cysteine in the presence of each other demonstrates that the two metal ion binding sites must be different on the α-subunit as well as on S-100a protein.

VI. CHEMICAL MODIFICATION STUDIES ON THE α-SUBUNIT OF S-100a PROTEIN.

Chemical modification provides one of the most useful methods for identifying functional groups of proteins. In addition, the technique can be used to examine the participation of individual residues in ligand binding or in the conformational change associated with ligand binding. As well, one can supplement the physical studies by determining whether chemical modification of the reactive side chains of particular residues affects the biological activities. To this end, some reagents are available for modifying the side chains of aspartic acid, glutamic acid, histidine, lysine, arginine, methionine, tryptophan, tyrosine and cysteine residues in a reasonably specific manner (Imoto and Yamada, 1989). In contrast, the recently developed technique of site directed mutagenesis results in the alteration of specific residues only, without undergoing any side reactions with other residues that have been known to occur with the majority of the chemical modification reagents. However, thc latter offer advantages over genetic methods in two aspects: (a) the modification is performed after the tertiary and quaternary structure of the protein is formed and (b) by varying the reaction conditions or increasing the modification with greater excesses of reagent, one can correlate the degree of modification of the amino acids with changes in the activities of the proteins. For example, this approach has been successfully applied to a study on bovine pancreatic deoxyribonuclease whereby chemical modification of 3 tryptophans led to elucidation of their respective roles in the catalytic activity of the enzyme (Sartin *et al.*, 1980).

S-100 protein was deemed to be a good candidate for chemical modification studies since Ca^{2+} binding to the α-subunit resulted in a gross conformational change and additionally affected the microenvironment of aromatic chromophores. The C-terminal region of the α-subunit is characterized by a single tryptophan (Trp 90) as well as the presence of one of the two tyrosines (Tyr 74). Moreover, these residues are located in close proximity to the putative Ca^{2+} binding site at positions 62 - 73 (Isobe and Okuyama, 1978). Since both the Trp and Tyr side chains contain reactive groups which are amenable to chemical modification, a study of the metal ion binding properties of the chemically modified α-subunit should yield additional information with regards to the nature of the observed conformational change .

A. Nitration of the α-subunit with tetranitromethane

Tetranitromethane (TNM) is a convenient reagent for the chemical modification of tyrosine residues in protein (Sokolovsky *et al.*, 1966). Nitration of tyrosine residues with TNM shifts the electronic transitions of the molecule from the near UV to the visible region of the spectrum (ε_{max} = 428 nm, at pH 8), and thus serves as an easily accessible probe of its microenvironment. Since the nitrotyrosyl chromophore may possess conformational or environmental asymmetry which can be detected by CD, modification of tyrosines with TNM offers an advantage over other techniques such as iodination or acetylation with N-acetylimidazole. The α-subunit is characterized by the presence of 2 tyrosines, at positions 26 and 74. Based on UV difference and CD spectroscopy, $Ca^{[illegible]+}$ binding to the α-subunit perturbs the microenvironment of the tyrosines. In this section, the tyrosines in the α-subunit have been chemically modified with TNM, in order to probe the effect of metal ion binding on the conformation of the protein in the vicinity of the tyrosines.

1. Spectrophotometric titration of the native α-subunit

Spectrophotometric titrations were performed in order to determine the ionization state of the 2 tyrosine residues of the α-subunit of the S-100a protein. Fig. 37 reveals the titration curves of the α-subunit in the absence and presence of Ca^{2+}. The experimental data can be described by the sum of 2 titration curves with estimated p*K*'s of ~ 10 and ~ 11 (in the absence and presence of Ca^{2+}). In both instances, the theoretical curves did not fit very well to the experimental data points at the beginning of the titrations, probably due to the effect of oxidized DTT in the medium, which possesses an absorption band in the 280

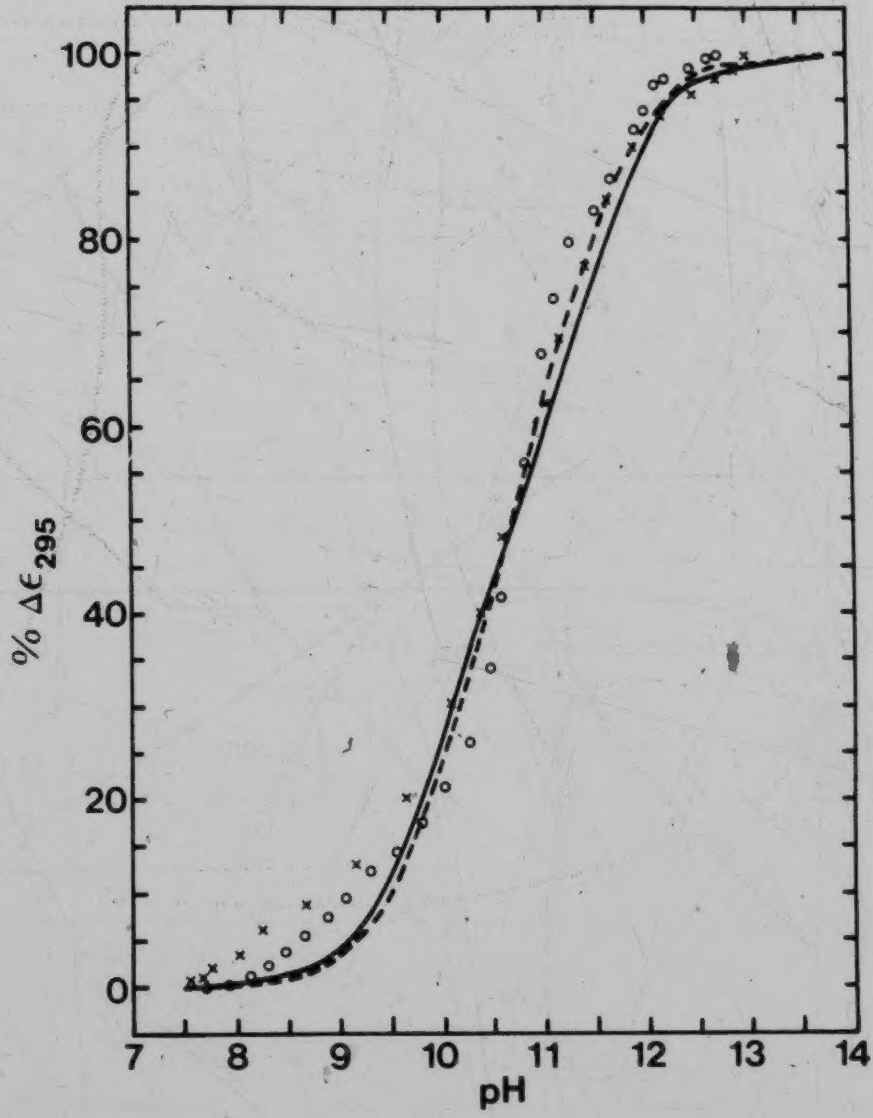

Fig. 37 Spectrophotometric titration of the α-subunit of S-100a protein. The percentage change in molar absorbance at 295 nm is plotted as a function of pH. The titrations were performed in the presence of 1 mM EGTA (o) or 1 mM Ca^{2+} (x), respectively, in a buffer containing 5 mM Tris/HCl, 50 mM NaCl, 1 mM DTT, pH 7.5. The points shown in the graph were determined experimentally. The lines are calculated according to the Henderson-Hasselbach equation, with p*K* values of ~ 10.0 and ~ 11.4, in the presence of 1 mM EGTA (-----) and 1 mM Ca^{2+} (——), respectively.

nm region (Cleland, 1964). The results obtained with the α-subunit were similar to those reported for the β-subunit of S-100b protein, where the single tyrosine at position 17 had an unusually high p*K* value of 11 - 11.5 (Mani *et al.*, 1983). Since far UV CD studies indicated that the tyrosine in S-100b ionized only after irreversible denaturation of the protein upon titration beyond pH 11, one should not give too much credence to the apparent p*K* values suggested by the titration curves shown here except to conclude that the 2 tyrosines in the α-subunit exist in different environments. The observation of a tyrosine with a high p*K* value could be due to a burial of the residue in the interior of the protein molecule or, alternatively, intramolecular interactions may exist between a phenolic hydroxyl group and a carboxyl group of either an aspartic or glutamic acid, as suggested by fluorescence studies with the homologous intestinal calcium binding protein (O' Neil *et al.*, 1982). An earlier UV difference study performed with the native α-subunit suggested that Ca^{2+} binding to the apoprotein caused one of the two tyrosines to become more exposed to solvent. However, based on the spectrophotometric titration data obtained in this study, the identity of which of the two tyrosines is perturbed in the presence of Ca^{2+} cannot be established.

2. Reaction of the α-subunit with TNM on an analytical scale

The reaction conditions for nitration were investigated on an analytical scale. The course of nitration was monitored spectrophotometrically at 428 nm, where the nitrophenolate ion absorbs maximally ($\varepsilon_{428\ nm}$ = 4,100 $M^{-1}cm^{-1}$, at pH 8) (Sokolovsky *et al.*, 1966). The increase in absorbance at this wavelength serves only as a semiquantitative guide to the true reaction course since it contains a small contribution from the trinitromethane anion (ε_{max} = 350 nm), a side reaction product formed during nitration. Absorbance is also dependent on the complete ionization of the nitrophenol produced in the reaction. When the α-subunit (3.5 x 10^{-5} M, in 50 mM Tris/HCl, pH 8.0) was nitrated in the presence of 110 fold molar excess of TNM, the rate of nitration was similar in the absence and presence of 1 mM Ca^{2+} (data not shown). Using an extinction coefficient of 4,100 $M^{-1}cm^{-1}$ for the nitrophenolate ion, the extent of nitration was estimated as 0.6 and 0.55 nitrotyrosine/mole subunit in the absence and presence of Ca^{2+}, respectively. These results suggest that the binding of Ca^{2+} to the apoprotein does not affect the rate of nitration of the tyrosine residues. The reaction was also performed at a lower molar excess of TNM (10:1), and resulted in the formation of 0.2 nitrotyrosine/α-chain based on a $\varepsilon_{381\ nm}$ value of 2,200 $M^{-1}cm^{-1}$ for the nitrotyrosine (Sokolovsky *et al.*, 1966), suggesting that the efficient nitration of tyrosines in the α-subunit requires a large excess of reagent.

3. Preparative scale reaction of the α-subunit with TNM

Preparative scale nitration of the α-subunit was performed with TNM at a reagent to protein ratio of 100:1, in 50 mM Tris/HCl, pH 8.0 and 1 mM EDTA. After the reaction, excess reagent was removed from the nitrated α-subunit by gel filtration and the product lyophilized from the pooled fractions. Since polymer formation had been observed in the reaction of proteins with TNM (Riordan and Sokolovsky 1971; Glazer, 1976), the modified α-subunit was subjected to analysis by SDS polyacrylamide gel electrophoresis (Laemmli, 1970). In Fig. 38A, it can be seen that, in the presence of 1 mM EDTA, most of the nitrated α-subunit (lane c) comigrated with the native protein (lane b). The latter is estimated to have a Mr of 10,000 ± 500 as deduced from its mobility in a 12.5% polyacrylamide gel, with reference to the mobilities of several standards. The standards are: (lane a) α-lactalbumin (Mr 14,200); trypsin inhibitor (Mr 20,100); trypsinogen (Mr 24,000); carbonic anhydrase (Mr 29,000); glyceraldehyde-3-phosphate dehydrogenase (Mr 36,000). However, in the nitrated sample, (lane c), there is a small amount of high molecular weight component which migrated with an estimated Mr of 20,500 ± 500 on the SDS gel, suggesting that a fraction of the α-subunit had dimerized after incubation with TNM. The high molecular weight component in lane (c) migrated ahead of a band

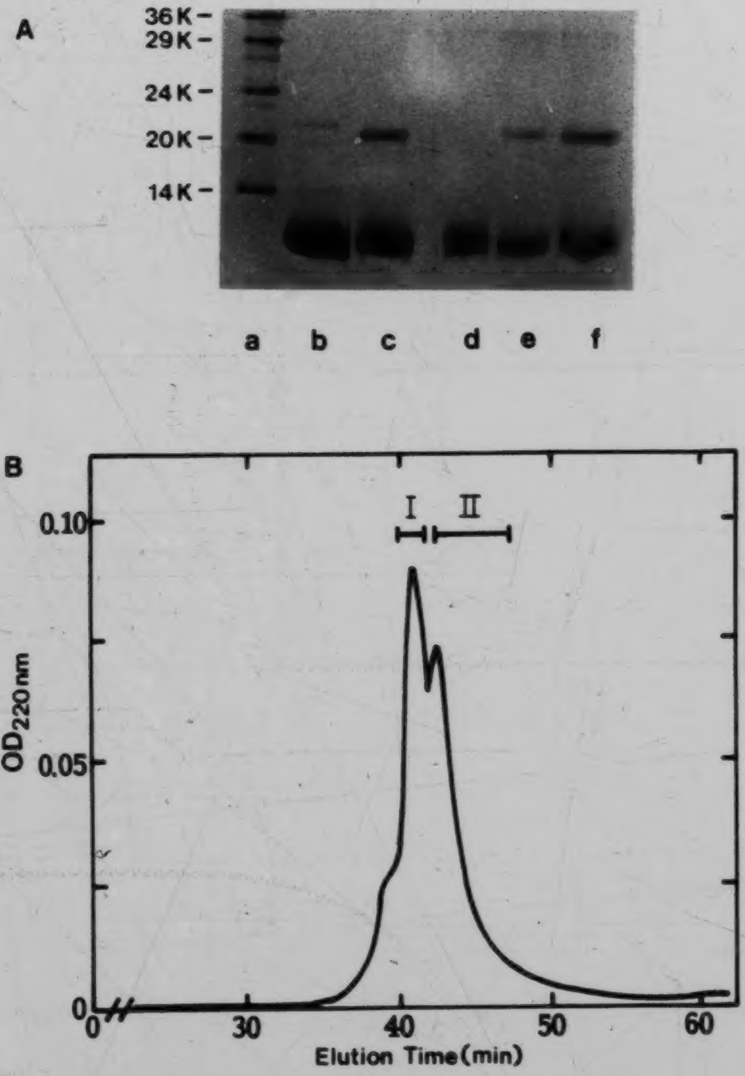

Fig. 38 (A) SDS polyacrylamide gel electrophoresis of the nitrated α-subunit in the presence of 1 mM EDTA. The samples were prepared by incubation at 95 °C for 3 mins in the presence of 2 % SDS and 50 mM DTT. The samples are: (a) molecular weight standards as described in the text; (b) native α-subunit; (c) nitrated α-subunit; (d) pooled fractions in peak I of C-18 column; (e) pooled fractions in peak II of C-18 column; (f) same as lane e but with twice the loading of the sample. (B) Chromatographic profile of the nitrated α-subunit from a Synchropak C-18 column (250 x 10 mm i.d.). The nitrated α-subunit was chromatographed under the following conditions: solvent A consisted of 0.1% TFA, 10% acetonitrile and solvent B was made up of 0.1% TFA, 60% acetonitrile, at 1% B/min gradient and at a flow rate of 3 ml/min at 25 °C. The fractions in peak I and II were pooled separately.

observed at a similar position in the native protein in lane (b). The latter was contaminated to a minor extent by a protein with an estimated M_r of 21,500 ± 500. In order to remove the dimerized material, the nitrated α-subunit was subjected to further purification by reversed phase HPLC. Fig. 38B shows the elution profile of nitrated α-subunit chromatographed on a C-18 column. SDS PAGE of the pooled fractions (Fig. 38A) revealed that the material recovered in peak I (lane d) was homogeneous and free from high molecular weight polymers while peak II (lanes e and f) contained a mixture of proteins heterogeneous in their molecular weights. The extent of nitration of the modified α-subunit recovered in peak I was spectrally determined ($\varepsilon_{381}{}^{M} = 2,200$), indicating the presence of 0.79 nitrotyrosine/ mole α-chain. Moreover, amino acid analysis performed with this sample revealed, that apart from the tyrosine, nitration of the α-subunit did not result in the modification of other amino acids such as methionines and histidines. Hence, this material (peak I) was used exclusively in the physico-chemical studies of the nitrated α-subunit reported in this section. However, owing to fact that the HPLC achieved only a partial separation of the nitrated α-subunit from the high molecular weight polymer, only a small amount of material was recovered for these studies. Low speed sedimentation equilibrium experiments performed with the nitrated α-subunit (peak I) under native solvent conditions gave a weight average molecular weight ($\overline{M}_w$) distribution of 11,800 to 18,800 ± 1000. Since the study was performed using a relatively low protein concentration (0.4 mg/ml), it is possible that the observed range of weight average molecular weights could be due to a concentration effect. In the case of the unmodified protein, which exists as dimers in native solvents, the protein has an increasing tendency to undergo dissociation when the concentration is below 1 mg/ml. Alternatively, the possibility exists that the introduction of a bulky nitrotyrosyl group could destabilize the quaternary structure of the homodimer.

4. Spectroscopic studies on the nitrated α-subunit

(i) UV absorption spectroscopy. The UV absorption spectrum of the metal free nitrated α-subunit (0.79 nitrotyrosine/mole protein) is shown in Fig. 39. The spectrum

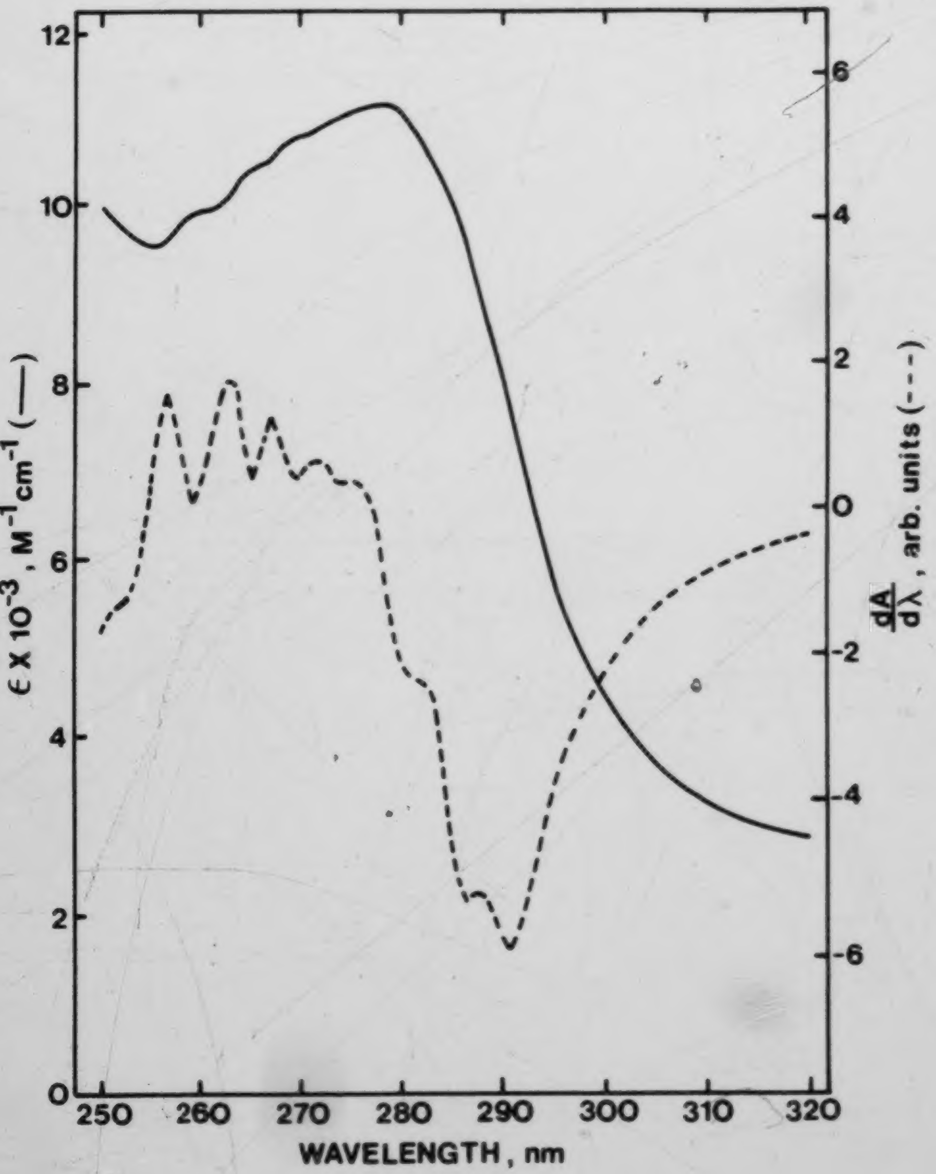

Fig. 39 UV absorption (——) and derivative (------) spectra of the nitrated α-subunit (62 μM) in 0.1 M Tris/HCl, 15 mM 2-mercaptoethanol, pH 7.5, in the absence of metal ions.

was measured at pH 7.5, so the nitrotyrosyl residues, which have an apparent p*K* value of approximately 7, would be partially ionized (Riordan and Sokolovsky, 1971). The absorption spectrum in the visible region is characterized by a broad band at 410 nm (not shown) which reflects the presence of the o-nitrophenol group. In the near UV region (250-320 nm), the spectrum of the nitrated protein is very similar to the native α-subunit (previously shown in Fig. 10a), but exhibits a significant increase in absorbance. At 278 nm, the nitrated α-subunit has a molar absorptivity of 11,155 M^{-1} cm^{-1}, compared to a value of 8,300 M^{-1} cm^{-1} observed for the native protein. The pronounced hyperchromicity is due to the more intense electronic transitions that occur in nitrotyrosyl chromophores compared to tyrosines (DiBello and Griffin, 1975). To emphasize the fine structure induced by the phenylalanine residues in the 260 nm region, a derivative spectrum of the nitrated α-subunit is also shown (Fig. 39), and clearly demonstrates the constancy in wavelength position of these bands. The single tryptophan that distinguishes the α-from the β-subunit appears to be unaffected by nitration as evident by the presence of a minimum at 292 nm. The appearance of 2 shoulders at 280 nm and 286 nm could be due to absorption by the unsubstituted tyrosine, since 2 minima are also observed at the corresponding positions in the derivative spectrum of the native α-subunit.

(ii) Circular dichroism

Upon chemical modification of a protein, it is imperative to ascertain whether or not the secondary structure of the protein has been altered. Consequently, far UV CD measurements were performed on the nitrated α-subunit to determine if the modified protein has undergone any significant conformational change. In the absence of metal ions, the nitrated α-subunit has a $[\theta]_{222}$ value of -12,711 ± 300 deg.cm^2.$dmol^{-1}$ (Fig. 40) in contrast to a value of -17,000 ± 300 deg.cm^2.$dmol^{-1}$ observed for the native protein (spectrum not shown). Analysis of the CD spectra by the method of Chen *et al.* (1974) revealed that the nitrated protein has an α-helical content of 42% compared to a value of 56% observed in the native protein. Moreover, CD measurements performed with a control

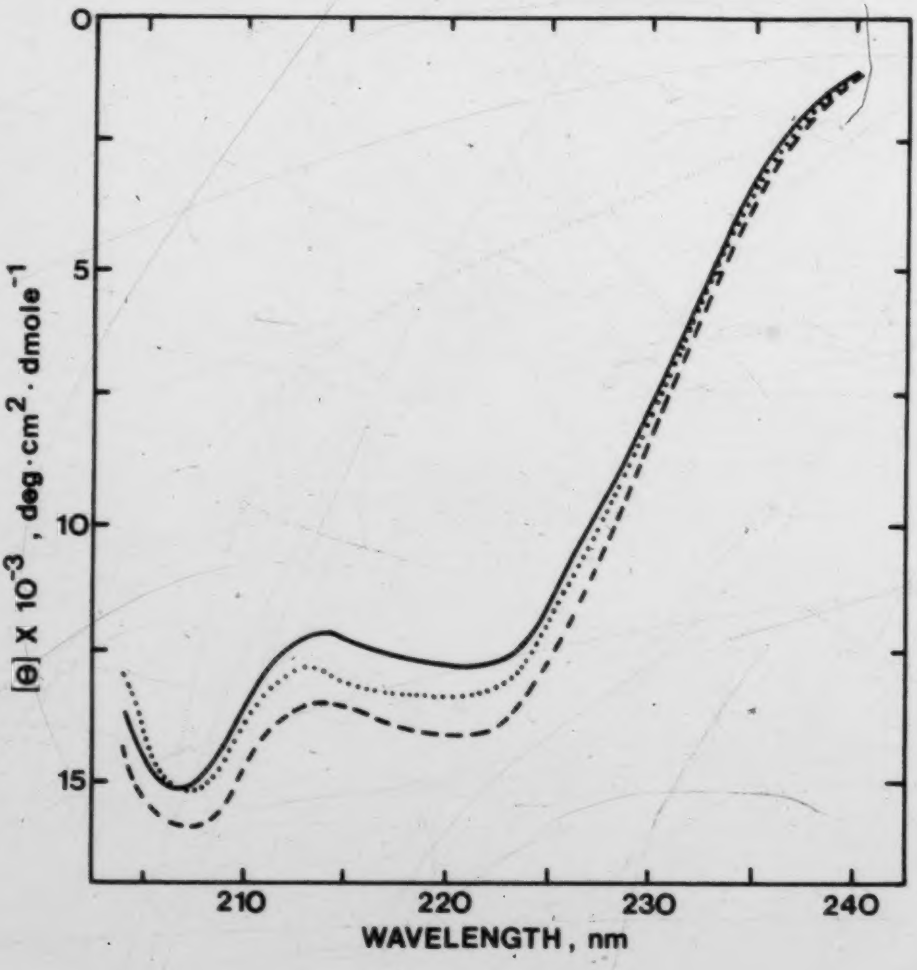

Fig. 40 Far UV CD spectra of the nitrated α-subunit in 0.1 M Tris/HCl, 15 mM 2-mercaptoethanol, pH 7.5, in the absence of metal ions (——); in the presence of 1 mM Ca^{2+} (-----), and in the presence of 0.2 mM Zn^{2+} (·······). The concentration of the protein was 24 μM.

sample that had been subjected to a similar treatment as its nitrated counterpart, apart from the omission of TNM, did not reveal any significant change in the $[\theta]_{222}$ value from that of the native protein. The results suggested that the mild conditions used in the nitration reaction are unlikely to affect the structural integrity of the protein and that the modification of the tyrosine residues is responsible for altering the conformation of the polypeptide backbone of the nitrated protein. Since the nitrated protein still retained a significant amount of secondary structure, a decision was made to further explore the effect of metal ion binding on the conformation of the nitrated protein. Addition of 1 mM Ca^{2+} to the nitrated α-subunit caused the $[\theta]_{222}$ value to increase by 10% to -14,015 ± 300 $deg.cm^2.dmol^{-1}$. When 0.2 mM Zn^{2+} was subsequently added to the same sample, the ellipticity decreased to give a final $[\theta]_{222}$ value of -13,320 ± 300 $deg.cm^2.dmol^{-1}$ (Fig. 40). Analysis of the CD data suggested the α-helical content to rise from 42% to 45% in the presence of Ca^{2+} and then decreased to 43% when Zn^{2+} was subsequently added. On the other hand, when the order of metal ion addition was reversed, addition of Zn^{2+} to the apoprotein resulted in the $[\theta]_{222}$ value to decrease by 7 % to -11,830 ± 300 $deg.cm^2.dmol^{-1}$ (spectrum not shown). When Ca^{2+} was subsequently added to the same sample, the ellipticity value increased to give a final value of -13,540 ± 300 $deg.cm^2.dmol^{-1}$. Hence, the CD results suggested that the order of addition of metal ion was not important, and that the final observed spectra were essentially the same. Upon comparing the metal ion binding properties of the nitrated α-subunit with those reported for the native protein, it is observed that Zn^{2+} exerted a similar effect on both proteins, namely a reduction in the α-helical content by 2 - 4%. In contrast, the Ca^{2+} effect on the nitrated protein was different from the native α-subunit. In the latter case, addition of Ca^{2+} caused the α-helical content to decrease from 56% to 51%. It is conceivable that nitration of the α-subunit, which resulted in alteration of the gross conformation of the protein, could lead to a modified α-subunit with Ca^{2+} binding properties different from those observed with the native protein.

The near UV CD spectrum of the nitrated α-subunit is shown in Fig. 41. In the absence of Ca^{2+}, the spectrum is characterized by a weak intensity band at 297 nm and fine structure in the 260 nm region. These CD bands were also observed in the aromatic spectrum of the native α-subunit (previously shown in Fig. 12) and had been assigned to the single tryptophan and one or more phenylalanines, respectively. However, the spectrum of the nitrated protein is distinguished by the presence at 277 nm, of only one of the two tyrosyl bands observed in the unmodified protein and, also differences in the ellipticity values throughout the 250-310 nm region. The CD bands in the 280 nm region of the spectrum of the nitrated α-subunit cannot be unequivocally assigned, since it is not clear whether the unsubstituted tyrosine and/or the nitrotyrosyl chromophore contributes to optical activity in this region of the aromatic spectrum. Moreover, the alteration in the conformation of the nitrated protein may exert an indirect effect on the asymmetric environment of these chromophores, resulting in a CD spectrum different from that observed in the native protein. CD measurements performed with the nitrated α-subunit in the visible region (320-500 nm) revealed that the optical activity was essentially zero (spectrum not shown), suggesting that the nitrotyrosyl chromophore may not be located in an asymmetric environment to give rise to Cotton effects. Addition of 1 mM Ca^{2+} to the nitrated α-subunit at pH 7.5, resulted in a perturbation of the tryptophan band at 297 nm and the CD bands in the 280 nm region. In addition, a sharpening of the phenylalanine fine structure was also observed. The Ca^{2+} effect on the microenvironment of the aromatic chromophores of the native protein has been reported previously (see Fig. 12). Essentially, Ca^{2+} has a larger effect on the environment of one or both tyrosines, resulting in on average, an 80% reduction in the $[\theta]_{277}$ value. In the case of the nitrated protein, a 38% decrease in the ellipticity value was observed at the corresponding wavelength. Since aromatic CD contributions are conformation dependent (Strickland, 1974), the discrepancy in the observed Ca^{2+} induced spectra between the nitrated and native protein may in part be due to the fact that nitration has altered the conformation of the protein On the other hand,

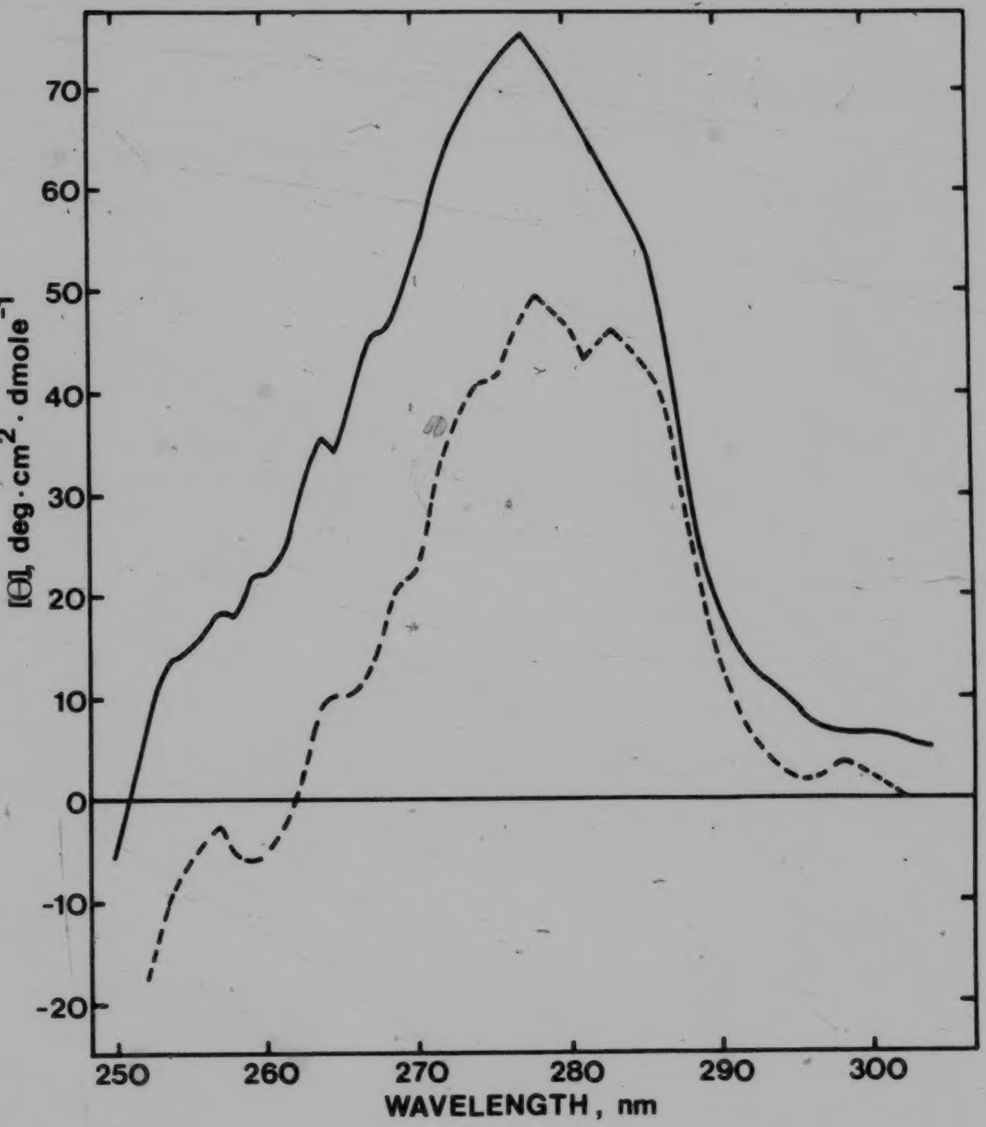

Fig. 41 Near UV CD spectra of the nitrated α-subunit in 0.1 M Tris/HCl, 15 mM 2-mercaptoethanol, pH 7.5, in the absence of Ca^{2+} (——), and in the presence of 1 mM Ca^{2+} (------). The concentration of the protein was 61 μM.

addition of Zn^{2+} to the nitrated protein only induced a small alteration in the region near 280 nm, resulting in a reduction of the $[\theta]_{277}$ and $[\theta]_{284}$ values by 6% and 10%, respectively (spectrum not shown). Zn^{2+} binding to the native α-subunit also induced a small perturbation in the environment near the tyrosine chromophore (Table VII). Furthermore, in the presence of Zn^{2+}, Ca^{2+} was able to exert an effect similar to that observed upon the addition of Ca^{2+} to the metal free nitrated α-subunit.

(iii) Fluorescence spectroscopy

Although the nitrotyrosyl chromophore lacks fluorescent properties, fluorescence measurements were performed with the nitrated α-subunit in order to determine whether the tryptophan at position 90 was affected by nitration. Of relevance was an earlier study which suggested that oxidation of tryptophan in papain by TNM resulted in inactivation of the enzyme (Morihara and Nagami, 1969). Upon excitation of the nitrated α-subunit at 280 nm, where both tyrosine and tryptophan absorbed, the emission maximum (λ_{max}) is centered at 340 ± 1 nm (Fig. 42). The latter, due to a partly buried tryptophan, was also observed in the native α-subunit which exhibited a λ_{max} of 339 ± 1 nm. Since the λ_{max} of the tryptophan generally reflects the polarity of its surrounding environment, the results suggested that the environment of the tryptophan in the nitrated α-subunit was indistinguishable from that of the native protein. The emission spectrum of the nitrated α-subunit is also characterized by a shoulder at 304 ± 1 nm, which is due to one or more unsubstituted tyrosines. An excitation maximum of 280 ± 1 nm was recorded for the nitrated protein when the emission was monitored at 340 nm. Addition of 1 mM Ca^{2+} at pH 7.5, resulted in a 15% decrease in the fluorescence intensity with no significant change in the position of emission maximum (Fig. 42), suggesting that the tryptophan was only minimally perturbed. The results are in contrast to those reported for the native protein where addition of Ca^{2+} resulted in a 2 nm red shift in the λ_{max} , to 341 ± 1 nm, accompanied by a 15% increase in the fluorescence intensity. The latter observation is consistent with the tryptophan becoming more exposed to solvent. Hence, Ca^{2+} affects the

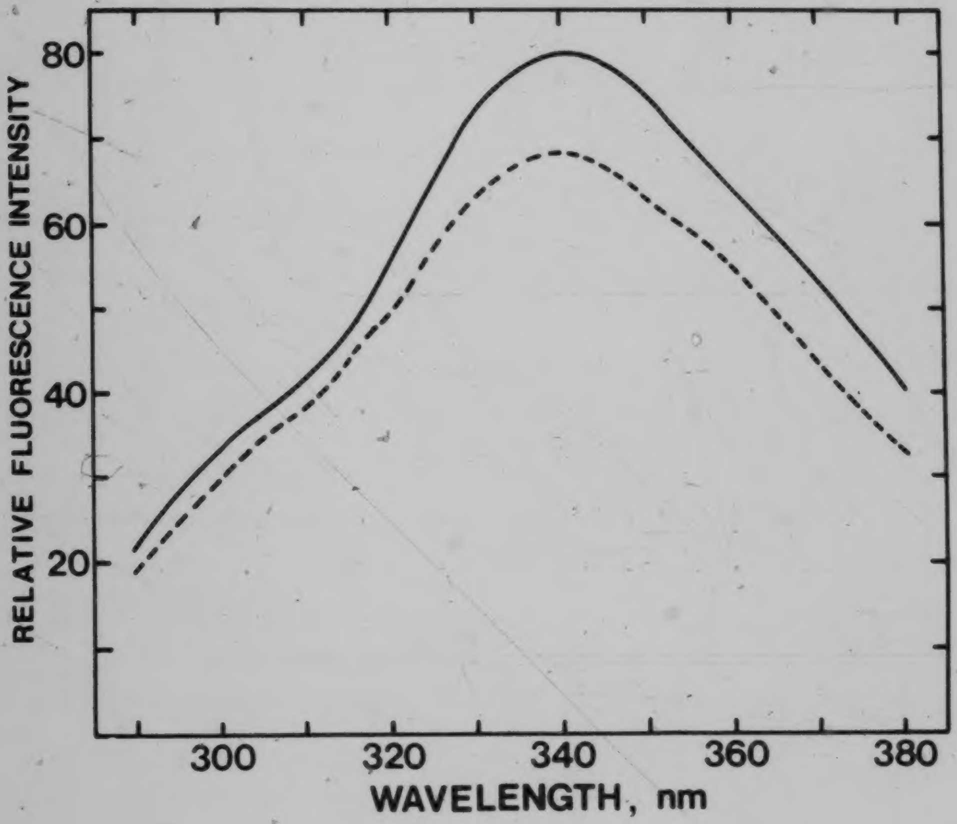

Fig. 42 Fluorescence emission spectra of the nitrated α-subunit in 0.1 M Tris/HCl, 15 mM 2-mercaptoethanol, pH 7.5, in the absence of (——) and presence of (------) 1 mM Ca^{2+}. The concentration of the nitrated protein was 2.4 μM and the excitation wavelength was 280 nm.

microenvironment of Trp 90 in the nitrated α-subunit in a manner different from that observed in the native protein, and this correlates with the far UV CD results whereby addition of Ca^{2+} to the nitrated protein resulted in a small increase in the α-helical content, compared to a decrease reported for the native protein. The effect of Zn^{2+} binding on the nitrated protein was not examined by fluorescence spectroscopy since the metal ion had no significant effect on the emission spectrum of the native protein, as reported earlier.

5. Discussion

Nitration of tyrosine residues with tetranitromethane has been used successfully to study the conformational change induced by Ca^{2+} binding to intracellular CaBP such as calmodulin (Richman and Klee, 1978; 1979; Richman, 1979; Walsh *et al.*, 1979; McCubbin *et al.*, 1979), and skeletal and cardiac Tn-C (McCubbin and Kay, 1975; McCubbin *et al.*, 1979). Moreover, in the case of calmodulin, nitration of the 2 tyrosine residues appeared not to affect its ability to stimulate cyclic nucleotide phosphodiesterase in the presence of Ca^{2+} (Walsh and Stevens, 1977; Richman and Klee, 1978) and thus allowed the nitrotyrosyl to serve as a probe in the study of the interaction of calmodulin with its target enzymes.

The α-subunit of the S-100a protein is characterized by 2 tyrosines at positions 26 and 74 whereas the β-subunit only contains 1 tyrosine, at position 17. Spectrophotometric titrations of the α-subunit in the absence of divalent cations revealed 2 p*K* values, suggesting that the 2 tyrosines exist in different microenvironments. According to Nozaki and Tanford (1967), the usual p*K* range for a fully exposed tyrosine residue in a protein framework lies between 9.5 - 10.0. Hence, this suggests that one of the tyrosines (p*K* ~ 10) in the α-subunit is partially exposed. In contrast, the other tyrosine is characterized by a high p*K* value, similar to that observed for Tyr 17 on the β-chain, which ionized only after denaturation of the protein at high pH (Mani *et al.*, 1983). The results obtained from the spectrophotometric titrations lend further support to the UV difference spectral data reported earlier (Chapter IV). In the latter, it was suggested that Ca^{2+} binding to the α-subunit probably resulted in the perturbation of one of the two tyrosine residues, based on the magnitude of the observed tyrosine difference peak at 294 nm. Spectrophotometric titrations of the α-subunit suggested that one of the tyrosines in the apoprotein was more accessible to hydroxyl ions. Hence it can be inferred from this study that Ca^{2+} binding to the α-subunit probably perturbs the environment of the more exposed tyrosine rather than a partial perturbation of both residues. In general, the p*K* value of tyrosine decreases upon moving to a more polar environment, hence, Ca^{2+} binding to the α-chain is likely to affect the p*K* of the perturbed tyrosine. Furthermore, due to its accessibility, it is possible that the more exposed tyrosine in the apoprotein preferentially reacted with TNM.

The homologous intestinal calcium binding protein, ICaBP, bears similarity to S-100β in that the single phenolic group does not ionize significantly below pH 12 (O'Neil *et al.*, 1982). Upon examining the three dimensional structure of ICaBP (Szebenyi *et al.*, 1981; Szebenyi and Moffat, 1986), O'Neil and Hoffman (1987) suggested that the presence of a hydrogen bond, between Tyr-13 and Glu-35, could be responsible for the high p*K* value of the tyrosine in this protein. Furthermore, Szebenyi and Moffat (1986) suggested the same Tyr-Glu pair is maintained in S-100β and this might conceivably account for the high p*K* value of Tyr 17β. Morero and Weber (1982) also proposed the existence of a tyrosyl-carboxylate hydrogen bond in a mixture of S-100 protein based on the UV difference spectrum generated between pH 7.5 and 3.5. In the case of the α-subunit, the tyrosine corresponding to Tyr-17β is replaced by a histidine residue. Despite the presence of a tyrosine at position 26, it is not clear whether Tyr-26α is capable of making the intramolecular interaction similar to that observed with Tyr-17 on the β-chain. The assignment of the tyrosines to their respective p*K* values is also complicated by the fact that the second tyrosine is located at the beginning of a stretch of hydrophobic residues (positions 74 - 90), which could elevate the p*K* value of this tyrosine if it was buried in the cluster. A third explanation could be related to the fact that Tyr-74α is located in close proximity to a cluster of negative charges from the side chains of carboxyl groups in the putative C-terminal Ca^{2+} binding site (residues 62 - 73). This could also contribute to a high p*K* value of this tyrosine (Wetlaufer, 1962). Richman and Klee (1978) suggested that the high p*K* value of ~ 11.9 observed for Tyr-138 in calmodulin could be due to its close proximity to a cluster of acidic residues.

In addition to reacting with tyrosine residues, nitration with a large molar excess of TNM at pH 8 also resulted in the oxidation of tryptophan residues (Muhlrad *et al.*, 1968; Sokolovsky *et al.*, 1970). In S-100α, both UV difference and fluorescence studies have shown that Ca^{2+} binding induced the partially buried tryptophan to become more exposed to solvent. Since preliminary studies had shown that the rate of nitration was not significantly affected by the presence of Ca^{2+}, the nitration of the α-subunit was performed in the presence of EDTA, in order to minimize the possibility that the tryptophan might be oxidized by TNM. Under these conditions, nitration appeared not to affect the microenvironment of Trp 90α, since the emission maximum of the nitrated protein occurred at the same wavelength as that observed in the native protein. In addition, TNM is also known to react with sulfhydryl groups in proteins which leads largely to the formation of disulfides and subsequent polymerization into high molecular weight products (Sokolovsky *et al.*, 1969). The α-subunit is characterized by a single cysteine at position 85 in close proximity to Trp 90. SDS polyacrylamide gel electrophoresis of the HPLC purified nitrated α-subunit showed the protein to migrate with a M_r of 10,000 ± 500 in a 12.5% gel and was free of high molecular weight products (Fig. 38A, lane d), suggesting that cysteine 85 was not modified by TNM nitration. On the other hand, it can be seen that in the nitrated sample before chromatography on a C-18 column (Fig. 38A, lane c), a fraction of the modified protein migrated with a M_r of 20,500 ± 500 in a 12.5% polyacrylamide gel, suggesting that TNM induced the polymerization of the nitrotyrosyl α-subunit to some extent. However, it is not clear whether the polymerization was caused by intermolecular coupling of tyrosine residues (Bruice *et al.*, 1969) and/or cysteine residues.

Nitration of the metal free α-subunit with a 50 fold molar excess of TNM / tyrosine residue resulted in the detection of 0.79 nitrotyrosine / α-chain and elicited a conformational change at the level of the backbone. Furthermore, CD studies performed on a nitrated sample containing 0.2 nitrotyrosine / mole protein (nitrated with a 5 fold excess TNM / tyrosine under identical conditions) revealed that the structure did not differ significantly from the native protein with respect to the average secondary structural content of the folded polypeptide. Hence, these results taken together, suggested that TNM induced an alteration in the gross conformation of the protein which was related to the extent of nitration of the protein. In this respect, the α-subunit is more sensitive to treatment with TNM than is with the β-subunit. Under similar conditions, nitration of the single tyrosine at position 17 of S-100β with a 100 fold molar excess of TNM resulted in no significant alteration in the conformation and aggregation state of the protein (Mani and Kay, 1986). Despite the ability of nitrotyrosyl S-100β to bind 0.9 mole Ca^{2+}/subunit, both far and near UV CD measurements revealed that Ca^{2+} binding did not induce any significant conformational change, in contrast to those observed in the native protein. Although Tyr 17β is not located near the C-terminal Ca^{2+} binding site, the results suggest that the tyrosine is essential for monitoring the Ca^{2+} induced conformational change. In contradistinction, Zn^{2+} binding to S-100b was not affected by nitration, suggesting that the nitrotyrosyl chromophore served as a useful probe in monitoring changes in the environment near the Zn^{2+} binding site, which probably involves histidine ligands at positions 15 and/or 25. In the case of the nitrated α-subunit, the effects of Ca^{2+} on the tertiary structure and Zn^{2+} on the secondary structure are similar to those observed in the native protein. However, the fact that nitration resulted in altering the gross conformation of the protein, complicates the interpretation of the spectral data. Attempts were made to identify the tyrosine residue(s) nitrated in the α-subunit to aid in the interpretation of the conformational change induced by metal ion binding. However, these were unsuccessful owing to the poor recovery of the peptides after chromatography on the HPLC. Based on the results of the spectrophotometric titrations of the native α-subunit, which suggests that the two tyrosines exist in different microenvironments, it can be reasoned that nitration with TNM in the absence of divalent cations probably resulted in the modification of one of the two tyrosines. The exposed tyrosine residue could in theory react preferentially with TNM, but the latter had been reported to also react with the side chain of buried tyrosyl (Glazer, 1976). However, the possibility that a partial nitration of both residues occurred, cannot be completely ruled out. In summary, despite the extensive sequence homology between the 2 subunits of the S-100 protein, nitration of the α-subunit resulted in properties different from those observed with the β-chain, and this could conceivably be due to subtle differences in the microenvironment of the aromatic groups between S-100α and S-100β.

B. Oxidation of the α-subunit with N-Bromosuccinimide

N-Bromosuccinimide (NBS) has been widely used as a protein modifying reagent. When used under carefully controlled conditions, NBS reacts preferentially with tryptophan resulting in the oxidation of the indole ring to an oxindolealanine moiety, accompanied by a shift in the wavelength of maximum absorbance from 280 to 250 nm (Spande and Witkop, 1967a). The accessibility of the tryptophan to reaction as a function of its exposure to solvent is well documented (Spande and Witkop, 1967c). Hence, the availability for reaction as well as the rate of reaction have been used as criteria for assignment of exposure of tryptophan residues. The modification of proteins with NBS has allowed the identification of Trp residues in the active site of enzymes (Spande and Witkop, 1967b; Sartin *et al.*, 1980) and the monitoring of the conformational change of proteins upon ligand binding (O' Gorman and Matthews, 1977; Teichberg *et al.*, 1970). The α-subunit is ideal for chemical modification studies since Ca^{2+} binding affects the microenvironment of the single tryptophan (90α) located at the C-terminus of the molecule and oxidation of proteins with NBS can be performed under relatively mild conditions. In this section, the Ca^{2+} binding properties of the chemically modified α-subunit are examined by spectroscopic techniques.

In addition to reacting with tryptophan, NBS also oxidizes sulfhydryl groups at a much faster rate than tryptophan residues (Means and Feeney, 1971). For this reason, the single cysteine located at position 85 of the α-subunit was reacted with the thiol specific reagent, DTNB, before modification with NBS. The reaction was carried out in the presence of 8 M urea, 0.1 M Tris/HCl, pH 8.0 and under these conditions 0.7 SH group/subunit was estimated to have reacted, based on a $\varepsilon_{412\ nm}$ value of 14,290 $M^{-1}\ cm^{-1}$ for the thionitrobenzoate anion (Gething and Davidson, 1972). NBS has also been reported to oxidize tyrosine residues but at a slower rate than Trp (Means and Feeney, 1971). The oxidation of the sulfhydryl modified α-subunit was performed in 8 M urea, 0.1 M MES, pH 5.5. Under these conditions, the oxidizing species, N-Bromourea, displayed a greater specificity for Trp (i.e. to the exclusion of Tyr) (Funatsu *et al.*, 1964). Even though the NBS reaction is usually performed at pH 3 - 4, a pH closer to neutrality was used in this study in order to minimize the possibility of peptide bond cleavage (Spande *et al.*, 1966). Approximately 60% of the tryptophan was oxidized after reacting with a 10 fold excess of NBS, based on the decrease in the absorbance at 280 nm. The sulfhydryl was regenerated by treatment with 2-mercaptoethanol in the presence of denaturants, following the removal of excess reagent by dialysis. Amino acid analysis performed on the modified protein revealed good agreement with that of the native protein (results not shown) , suggesting that the side chains of the 2 histidines, 2 tyrosines and 2 methionines were not affected by NBS oxidation. SDS polyacrylamide gel electrophoresis indicated that the modified α-subunit migrated as a single band with an estimated M_r of 10,000 ± 500 in a 12.5% gel (data not shown). Low speed sedimentation equilibrium studies performed with the oxidized α-subunit (at 1 mg/ml) under native solvent conditions gave a weight average molecular weight ranging from 11,600 to 20,860 ± 1000 and 21,000 to 31,300 ± 1000 in the absence and presence of Ca^{2+}, respectively. The discrepancy observed in the $\overline{M}_w$ of the oxidized protein, in the absence of Ca^{2+}, could be due to a concentration effect whereby below 1 mg/ml , there was an increasing tendency for the dimeric αα to dissociate into monomers. On the other hand, Ca^{2+} appeared to stabilize the oxidized protein and to induce a slight aggregation.

1. Absorption spectrum of the NBS oxidized α-subunit

The absorption spectrum of the modified protein, in the absence of divalent cations, is shown in Fig. 43. It can be seen that the oxidation of the tryptophan resulted in a blue shift of the absorption maximum from 278 to 250 nm region. In addition to a strong absorption band at 250 nm, the oxindolealanyl chromophore also absorbs in the 280 nm region with moderate intensity (Conforth *et al.*, 1951). Hence, despite the fact that ~ 60% of the Trp was oxidized, the ε_{278} value of the NBS modified protein, 5,541 M^{-1} cm^{-1}, did not decrease significantly from the value of 8,300 M^{-1} cm^{-1} reported for the native protein (Masure *et al.*, 1984). In order to emphasize the fine structure that is characteristic of the absorption spectrum of phenylalanine chromophores in the 260 nm region, a derivative spectrum is also shown (Fig. 43) and clearly demonstrates the constancy in wavelength position of these bands. The derivative spectrum is also characterized by an extremum at 287.5 nm which is probably due to the tyrosines. Moreover, the absence of a trough at 292:5 nm suggested that NBS was effective in oxidizing Trp 90 which contributed to an absorption band at this wavelength in the spectrum of the native protein.

2. Circular dichroism

In order to ascertain whether oxidation of the Trp resulted in an alteration of the structural integrity of the α-subunit, far UV CD measurements were performed on the modified protein (Fig. 44). In the absence of metal ions, the NBS modified α-subunit has

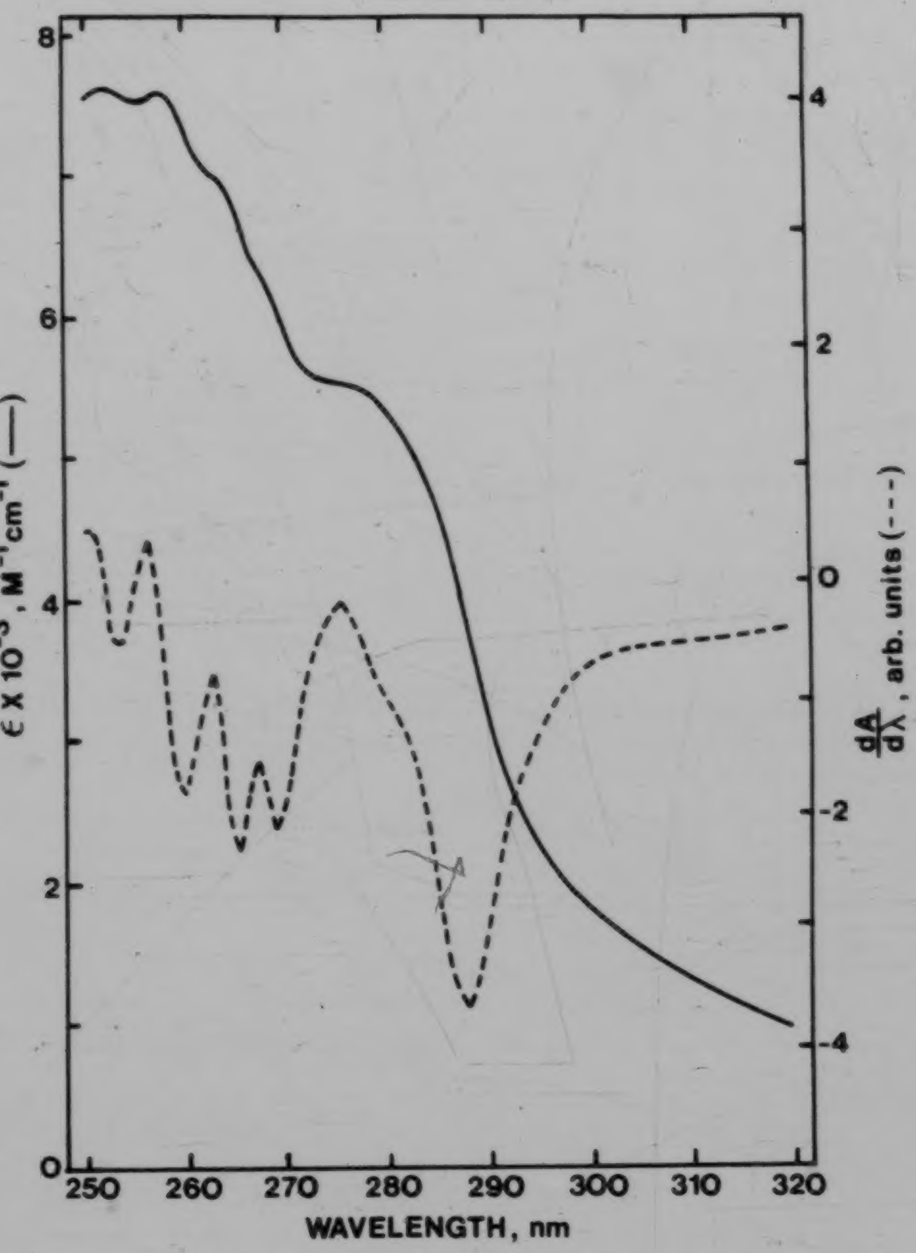

Fig. 43 UV absorption (——) and derivative (------) spectra of the NBS oxidized α-subunit in 0.1 M Tris/HCl, 1 mM DTT, pH 7.5, in the absence of metal ions. The concentration of the protein was 55 μM.

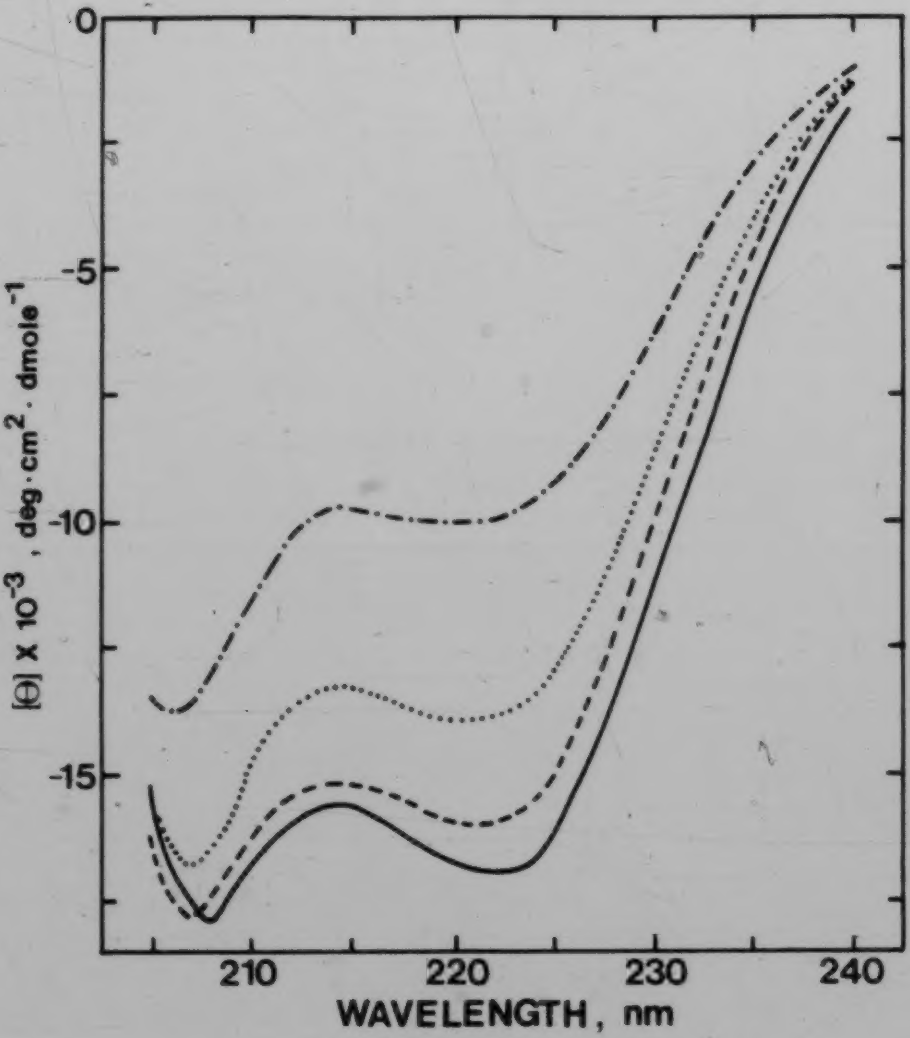

Fig. 44 Far UV CD spectra of the NBS modified α-subunit (55 μM) in 0.1 M Tris/HCl, 1 mM DTT, pH 7.5, in the absence (— · — ·) and presence of 1 mM Ca^{2+} (·······). Also shown are the spectra of the α-subunit (39 μM) in which the single sulfhydryl has been modified by DTNB and subsequently regenerated by treatment with 2-mercaptoethanol, with (——) and (------) representing the absence and presence of 1 mM Ca^{2+}, respectively, in a buffer containing 0.1 M Tris/HCl, 1 mM DTT, pH 7.5.

a $[\theta]_{222}$ value of -9,911 ± 300 deg.cm^2.dmol^{-1}, in contrast to a value of -16,430 ± 300 deg.cm^2.dmol^{-1} observed for the native protein (spectrum not shown). Since the oxidation of the α-subunit was performed with a sample of S-100α in which the sulfhydryl had been previously modified with DTNB and then subsequently regenerated after oxidation, CD measurements were also made on a control sample that had been subjected to identical treatment as its oxidized counterpart, apart from the omission of NBS. The result (Fig. 44) shows that the observed $[\theta]_{222}$ value for this sample, -16,880 ± 300 deg.cm^2.dmol^{-1}, is similar to that observed in the native protein, suggesting that the modification and the subsequent regeneration of Cys 85α is unlikely to cause a modification in the protein conformation. Hence, it can be reasoned that the oxidation of the single tryptophan in the α-subunit by NBS elicited a conformational change at the level of the backbone resulting in the α-helical content decreasing from 54% to 34 % as revealed by an analysis of the CD spectrum. Addition of 1 mM Ca^{2+} to the oxidized protein caused a 40% increase in the $[\theta]_{222}$ value to -13,890 ± 300 deg.cm^2.dmol^{-1} and the helical content to rise to 46%. In the case of the native protein and the sulfhydryl regenerated α-subunit, a 5.5% decrease in the $[\theta]_{222}$ value was observed upon the addition of Ca^{2+} to both samples, suggesting that the reversible modification of Cys 85 did not affect the Ca^{2+} binding properties of the protein.

Near UV CD measurements were also performed on the NBS modified α-subunit in the absence and presence of Ca^{2+}. In Fig. 45, it can be seen that although the general shape of the oxidized protein spectrum resembles that of the native protein, a marked decrease in the aromatic side chain Cotton effects in 270 - 287 nm region is observed in the modified protein. The CD bands at 278 and 284 nm region of the spectrum have been previously assigned to tyrosyl side chains. The large decrease in the ellipticity values of the CD bands in this region could be due to an alteration in the conformation of the NBS modified protein, exerting an indirect effect on the asymmetric environment of the tyrosyl chromophores, resulting in a CD spectrum different from that of the native protein.

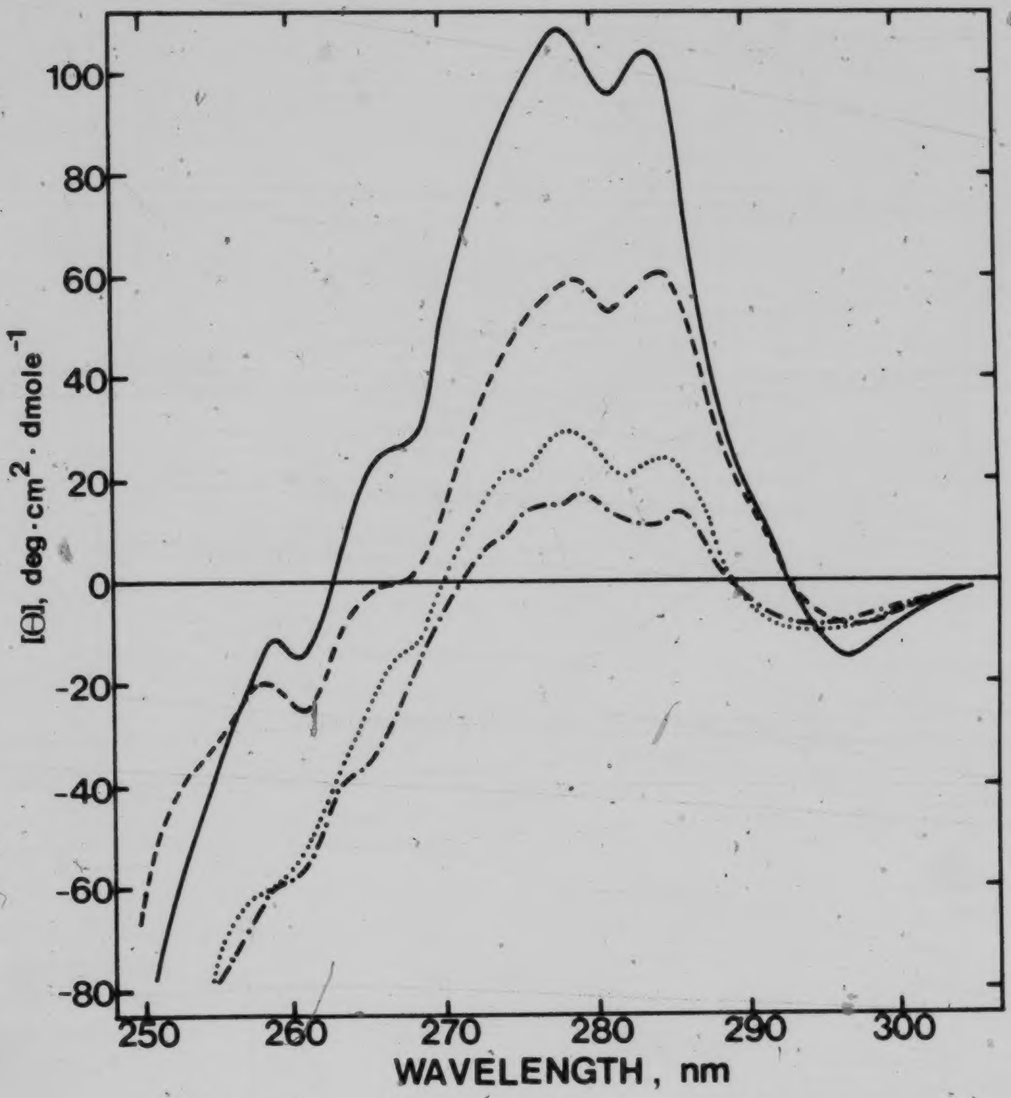

Fig. 45 Near UV CD spectra of the NBS modified α-subunit (55 μM) in 0.1 M Tris/HCl, 1 mM DTT, pH 7.5, in the absence (— · — ·) and presence of (·······) of 1 mM Ca^{2+}. The spectra of the native protein (62 μM) in the absence of (——) and presence of (------) 1 mM Ca^{2+}, in a buffer containing 0.1 M Tris/HCl, 1 mM DTT, pH 7.5 are also shown.

Moreover, NBS oxidation of the α-subunit also affected the ellipticity values of the phenylalanyl bands in the 260 nm region as well as the appearance of a shallow trough centered at 294 nm. Addition of 1 mM Ca^{2+} to the protein only resulted in a small increase in the ellipticity values around 280 nm, in contrast to a 50% reduction observed in the native protein. Furthermore, the reversible modification of Cys 85 did not appear to affect the tertiary structure or the magnitude of the Ca^{2+} induced spectral changes, since its near UV CD spectra, both in the absence and presence of Ca^{2+}, were indistinguishable from those of the native protein (spectra not shown).

3. UV Difference spectroscopy

The Ca^{2+} induced spectral changes in the microenvironment of the NBS modified α-subunit were also examined by absorption difference spectroscopy, a sensitive technique that can detect minor perturbations in the absorption spectrum. The Ca^{2+} induced difference spectrum of the oxidized α-subunit (Fig. 46) reveals a red shift in the difference peak at 286 nm , suggesting one or more of the tyrosines in the modified protein move to a less polar environment. Alternatively, the small change in the $\Delta\varepsilon_{286}$ value of 161 can be interpreted as an electrical charge induced change, as has been observed in ribonuclease where the $\Delta\varepsilon_{287}$ value was of the order of 60 (Donovan, 1965). On the other hand, the perturbations in the 250 nm region could be due to the oxindolealanine residue since it possesses an absorption maximum in this region (Conforth *et al.*, 1951). From Fig. 46, it can be seen that the Ca^{2+} effect on the microenvironment of the oxidized protein is minimal compared to the large spectral changes observed in the native protein. Moreover, the large difference peak centered at 292.5 nm, which has been assigned to Trp 90 in the native α-subunit, is absent from the Ca^{2+} induced spectrum of the modified protein, suggesting that NBS was effective in oxidizing the Trp. UV difference spectroscopy was also performed with the α-subunit in which Cys 85 was regenerated after DTNB modification, and resulted in a Ca^{2+} induced spectrum similar to that observed for the native protein.

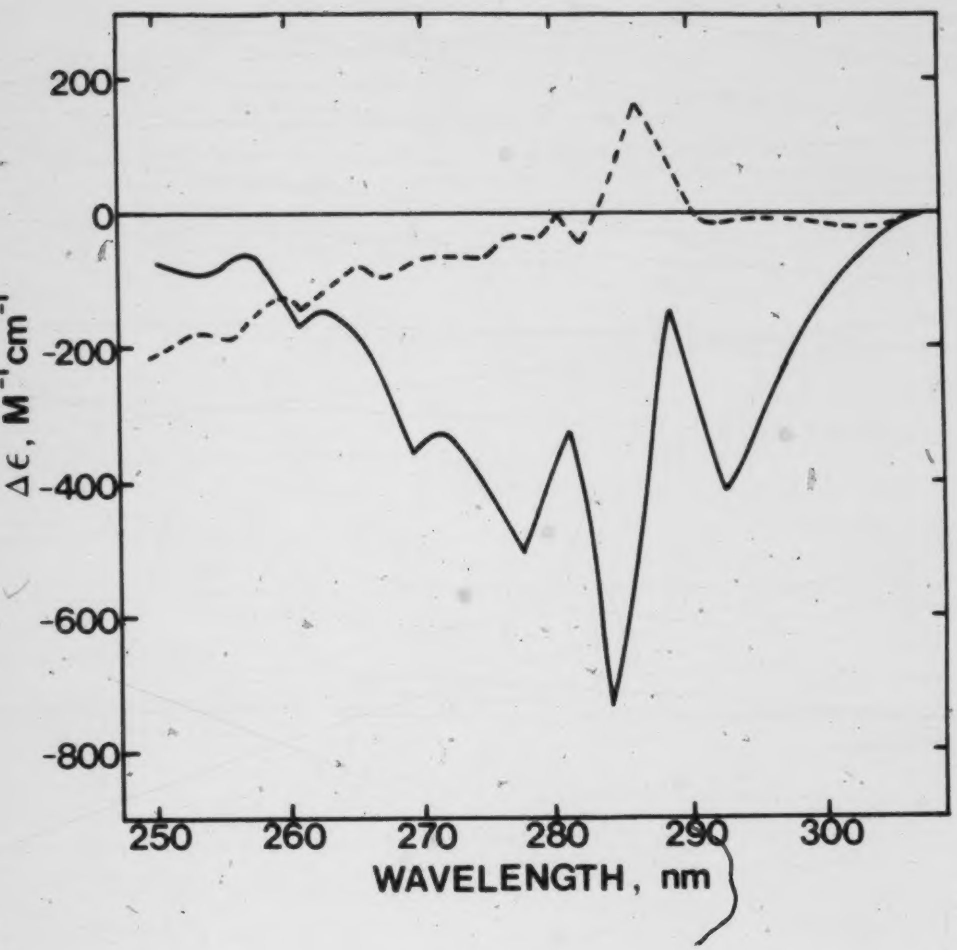

Fig. 46 Ca^{2+} induced UV difference spectra of the NBS oxidized α-subunit (------) and of the native protein (——), in 0.1 M Tris/HCl, pH 7.5. The concentrations of the proteins were 61 and 20 μM, respectively. Both spectra were generated by the addition of 2 mM Ca^{2+} to the apoprotein.

4. Fluorescence spectroscopy

Upon excitation of the NBS modified α-subunit at 280 nm, the emission maximum is centered at 302 ± 1 nm (Fig. 47), which is typical of tyrosine residues. This is in contrast to the λ_{max} of 339 ± 1 nm observed for the native protein. The latter is due to a buried tryptophan and owing to its high absorption (ε_{max}) and large quantum yield (Φ_F) properties, the fluorescence from this residue invariably dominates those of tyrosines in proteins. The results observed here suggested that the oxidation of Trp 90 to oxindolealanine removed most of the Trp influence on the emission spectrum of the α-subunit and revealed the fluorescence properties of the 2 tyrosine residues. When the excitation wavelength was set at 295 nm, i.e. selectively exciting the Trp residue, no significant emission was observed from the NBS modified protein in the wavelength region of 305 - 400 nm (spectrum not shown). Upon excitation at 280 nm, addition of 1 mM Ca^{2+} to the oxidized protein resulted in a 17% decrease in the fluorescence intensity, suggesting that the microenvironment of one or both of the tyrosine(s) is affected. Alternatively, the Tyr fluorescence could be quenched due to a proton transfer from tyrosine to an acceptor on the side chain of an amino acid brought into close proximity to the tyrosine upon Ca^{2+} binding, as suggested for ICaBP (O'Neil *et al.*, 1982). In the presence of Ca^{2+}, a small shoulder is observed in the 340 nm region of the emission spectrum of the NBS modified α-subunit. The latter is most likely due to the effect of Ca^{2+} on the unoxidized tryptophan, which only amounts to a small fraction since the "sensitivity" parameter (i.e. $\varepsilon_{max} \cdot \Phi_F$) for tryptophan is 5 fold greater than that of tyrosine (1100 and 200, respectively) (Cantor and Schimmel, 1980). Irrespective of whether Ca^{2+} is present or not, the excitation maximum of the NBS oxidized α-subunit, with the emission monitored at 303 ± 1 nm, exhibits a maximum at 279 ± 1 nm (Fig. 47), which is 3 nm blue shifted compared to the excitation maximum of 282 ± 1 nm of the native protein. The effect of Ca^{2+} binding on the fluorescence properties of the NBS modified α-subunit was different than that observed in the native protein and the Cys 85 regenerated α-subunit.

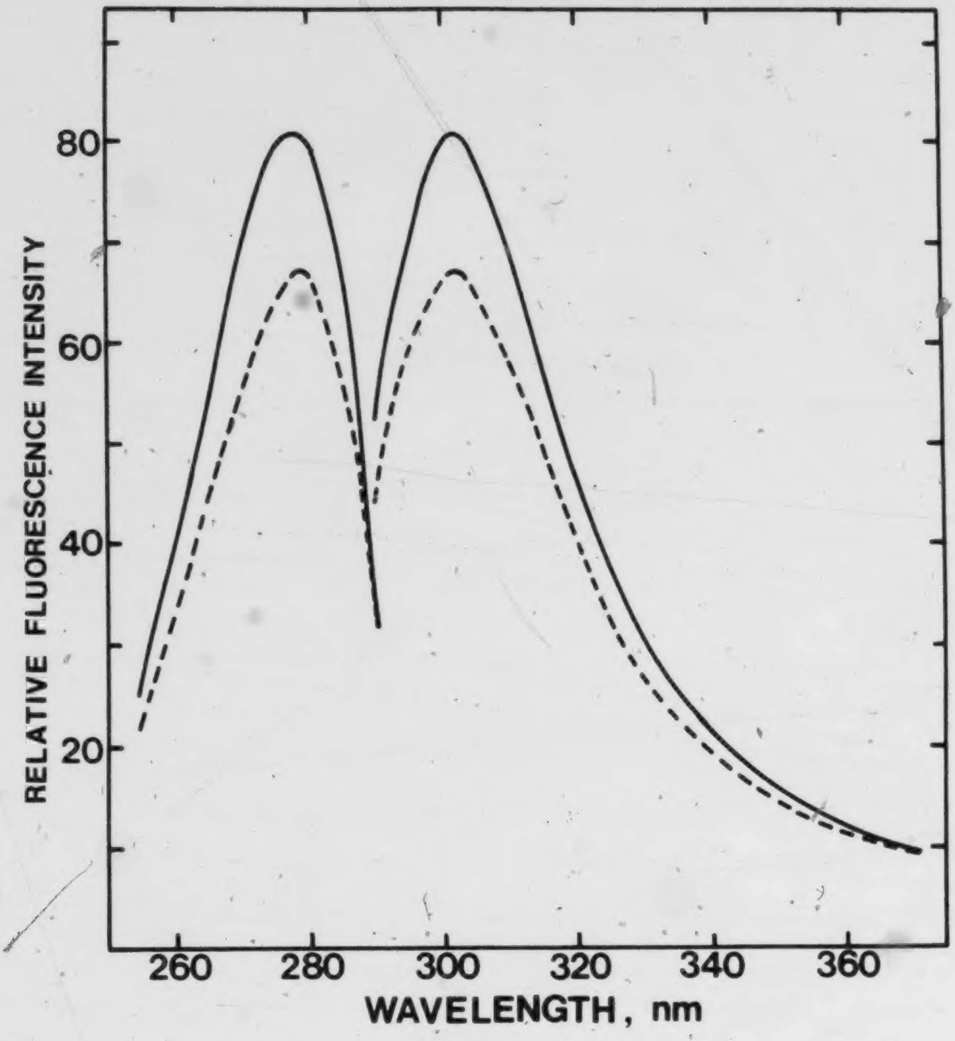

Fig. 47 Fluorescence excitation and emission spectra of the NBS modified α-subunit in 0.1 M Tris/HCl, 1 mM DTT, pH 7.5, in the absence (——) and presence of (------) of 1 mM Ca^{2+}. The emission spectra were monitored using an excitation wavelength of 280 nm. The excitation spectra were recorded when the emission maximum was set at 303 nm. The concentration of the protein was 2.8 μM.

5. Discussion

In this study it has been shown that NBS served as an effective reagent in the oxidation of the single tryptophan at position 90 of the α-chain. The reaction, performed in the presence of 8 M urea, did not affect the two tyrosine residues of the α-subunit. The modification of Cys 85 with DTNB also minimized the oxidation of the sulfhydryl group. Moreover, the subsequent regeneration of this cysteine with mercaptans did not affect the structural integrity or the Ca^{2+} binding properties of the α-subunit. Despite these precautions, treatment of the α-subunit with NBS resulted in altering the conformation of the protein at the level of the backbone and thus complicated the interpretation of the Ca^{2+} induced conformational changes revealed by the spectroscopic studies. Moreover, the tertiary structure in the environment of one or more of the tyrosines may have been affected and resulted in Ca^{2+} induced changes that are different from those observed in the native protein. Nevertheless, it can be concluded from this study that the tryptophan located at position 90α, situated in close proximity to the putative Ca^{2+} binding site (positions 62-73) (Isobe and Okuyama, 1978), is essential for monitoring the conformational change induced by metal ion binding in this region of the molecule.

In summary, the single tryptophan (Trp 90) and one or both tyrosines (Tyr 26 and 74) in the α-subunit have been selectively modified by oxidation with N-Bromosuccinimide and nitration with tetranitromethane, respectively. In both instances, the modifications have resulted in altering the gross conformation of the protein and led to a reduction of α-helical content. Based on the amino acid sequence (Isobe and Okuyama, 1981a), the tryptophan and one of the two tyrosines are located in a cluster of hydrophobic residues, between 74 to 90 on the α-chain, while the other hydrophobic cluster is located at the N-terminus, at positions 4 - 15. Since the earlier sedimentation equilibrium studies indicated that the α-subunit exists as homodimers linked together by non-covalent forces, this raises the possibility that hydrophobic forces may play a significant role in maintaining the tertiary and quaternary structures of the protein. It is tempting to speculate that the selective modification of Trp 90 and possibly the tyrosine at position 74 at the C-terminus, may lead to a partial disruption of the hydrophobic contacts and results in alteration of the gross conformation of the protein. In this respect, tetranitromethane, a hydrophobic compound (Glazer, 1976) may react preferentially with Tyr 74 on the C-terminal hydrophobic cluster. Clearly these chemical modification studies have demonstrated that the folding of the polypeptide backbone of the α-subunit is highly sensitive to the integrity of these aromatic residues.

VII. ISOLATION OF ALDOLASE C FROM BOVINE BRAIN AND AN ENZYMATIC AND PHYSICO-CHEMICAL STUDY OF ITS PUTATIVE INTERACTION WITH S-100a PROTEIN

Despite numerous efforts the function of the S-100 protein system is still not known. A rational approach to elucidating the function of S-100 protein would be to isolate, identify and characterize intracellular S-100 binding proteins. Since a high level of S-100 protein is found in the brain, the screening of S-100 binding proteins from an extract of the brain should provide an ideal source in the detection of these proteins. In fact, Zimmer and Van Eldik (1986), using a gel overlay procedure, detected a protein from rat brain that bound to ^{125}I-S100β, which showed partial sequence homology with fructose-1,6-bisphosphate aldolase [E.C. 4.1.2.13]. Further studies using purified enzyme preparations suggested that both the α and β-subunit stimulated the *in vitro* activity of the aldolase isoenzyme specific to nervous tissue in a Ca^{2+} dependent manner. Since the results suggested that aldolase C could be a potential intracellular target of S-100 protein in the brain, it was anticipated that, by characterizing the molecular mechanism of S-100 interaction with the enzyme physico-chemically, a better understanding of the function of the S-100 protein in nervous tissues might be obtained. To this end, the brain isoform of aldolase, i.e. the C isoenzyme, was purified from bovine brain, and an attempt was made to investigate its interaction with S-100 using a combination of enzymatic, spectroscopic and affinity chromatographic approaches.

A. Isolation of aldolase from bovine brain

Fresh bovine brains were used as the starting material for the purification of aldolase, based on the procedure of Buczylko *et al.* (1983). Fig. 48 shows a purification scheme for the isolation of aldolase C, with all the operations performed at 4 °C. Bovine brains were homogenized in 40 mM Tris/HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol and 0.1 mM PMSF, pH 7.5. After centrifugation to remove the insoluble

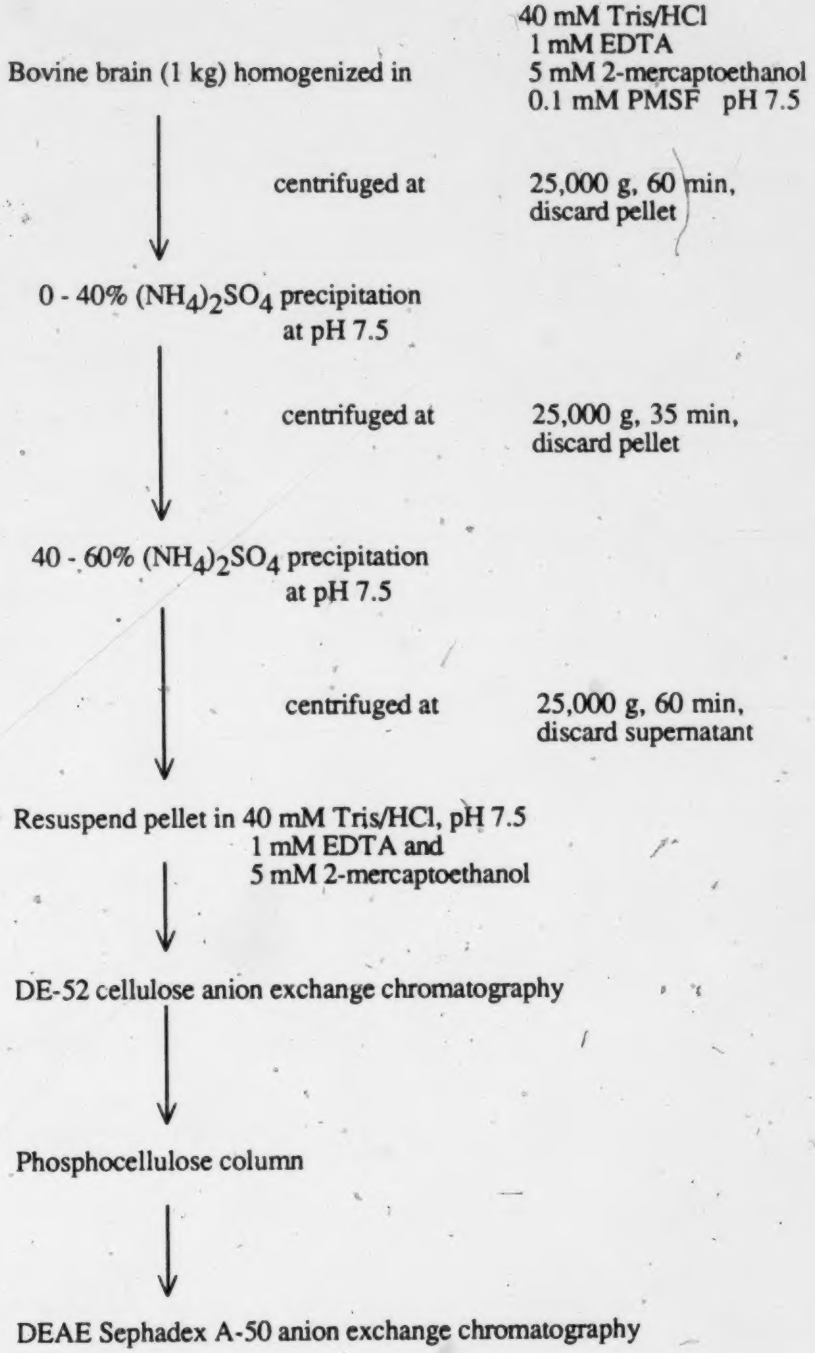
Bovine brain (1 kg) homogenized in
40 mM Tris/HCl
1 mM EDTA
5 mM 2-mercaptoethanol
0.1 mM PMSF pH 7.5
centrifuged at
25,000 g, 60 min, discard pellet
0 - 40% $(NH_4)_2SO_4$ precipitation at pH 7.5
centrifuged at
25,000 g, 35 min, discard pellet
40 - 60% $(NH_4)_2SO_4$ precipitation at pH 7.5
centrifuged at
25,000 g, 60 min, discard supernatant
Resuspend pellet in 40 mM Tris/HCl, pH 7.5
1 mM EDTA and
5 mM 2-mercaptoethanol
DE-52 cellulose anion exchange chromatography
Phosphocellulose column
DEAE Sephadex A-50 anion exchange chromatography

Fig. 48 Purification scheme of aldolase from bovine brain

material, the supernatant was brought to 40% saturation with respect to the ammonium sulfate concentration. After adjusting the pH to 7.5, the solution was left to stir for 30 min before clarification by centrifugation. The precipitate was discarded and the concentration of the ammonium sulfate in the supernatant was brought to 60% and, after adjusting the pH to 7.5, was left to stir for 3 hr. Following centrifugation, the pellet was then dissolved in a small volume of 40 mM Tris/HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5, and dialysed overnight against 2 changes of the same buffer. The dialysed protein was applied to a DE-52 cellulose column and after extensive washing with the starting buffer (40 mM Tris/HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5), the absorbed material was eluted using a linear gradient of 0 to 0.28 M NaCl . As seen in Fig. 49, aldolase activity can be detected in several peaks. Since aldolase, a tetrameric enzyme (Kawahara and Tanford, 1966), exists as a hybrid of A and C isoforms in the brain (Penhoet *et al.*, 1967), the peaks containing enzymatic activity represent the members of the hybrid A-C set of A_3C, A_2C_2, AC_3 and C_4, respectively. The homomeric A_4 isoform, which is positively charged at neutral pH, appeared not to bind to the DE-52 cellulose column (Penhoet *et al*, 1969a; Buczylko *et al.*, 1983). SDS PAGE (Fig. 52) revealed that a protein with a subunit M_r of ~ 40,000 is present in the peaks that contained enzymatic activity (lanes c, d, e, f, and g) and that the mobility of this protein in the gel coincides with that of a commercial preparation of muscle aldolase (lane b) which contained A_4 homomers (Penhoet *et al.*, 1969a). After pooling the fractions that contained aldolase activities from the DE-52 column, the protein solution was concentrated by ammonium sulfate precipitation (70% saturation, pH 7.5). The precipitated material was then centrifuged and the pellet dissolved in a small volume of 10 mM Tris/HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5, and dialysed overnight against 2 changes of this buffer. The protein was then applied to a phosphocellulose column and after extensive washing of the column, desorption of the enzyme was accomplished by affinity elution using the substrate, fructose-1,6-bisphosphate (2.5 mM). As seen in Fig. 50, the enzyme eluted as a sharp peak from the

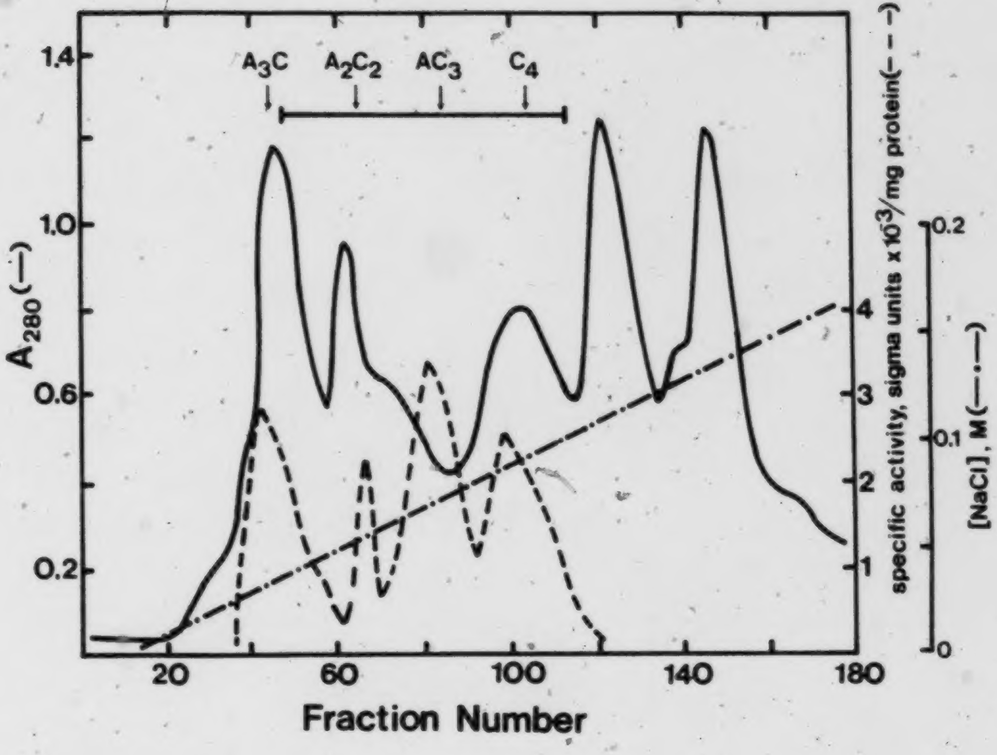

Fig. 49 DE-52 chromatography of bovine brain aldolase. The 40 - 60% ammonium sulfate fraction was dialysed against 40 mM Tris/HCl, 1 mM EDTA and 5 mM 2-mercaptoethanol, pH 7.5. The protein was applied to a DE-52 cellulose column (43 x 3.1 cm) equilibrated with the same buffer. The column was washed extensively with the starting buffer until the A_{280} of the effluent was below 0.1. Elution of the absorbed material was carried out with 2.2 L of a linear NaCl gradient (0 - 0.28 M) in the same buffer. The flow rate was 52 ml/hr and fractions of 11 ml were collected upon the application of the gradient. (|——|) represents the fractions pooled containing the A-C hybrid set of A_3C, A_2C_2, AC_3, and C_4 aldolase isoenzymes.

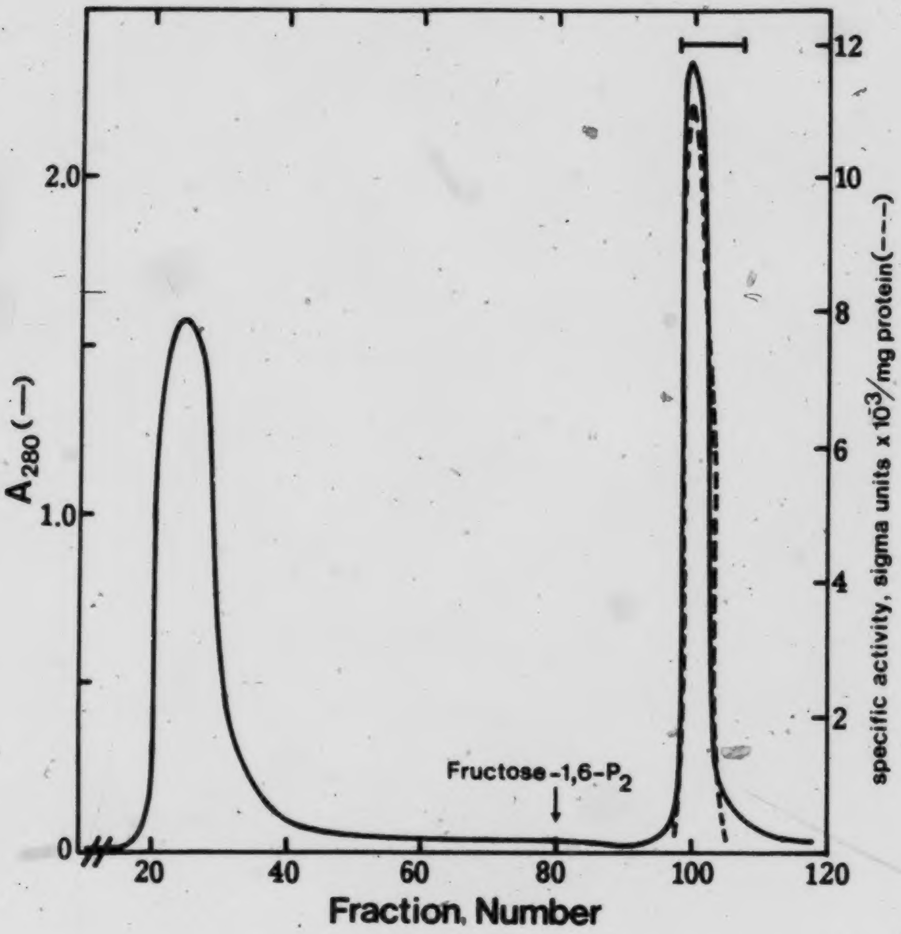

Fig. 50 Phosphocellulose chromatography of brain aldolase. The combined fractions of DE-52 cellulose column were concentrated by ammonium sulfate precipitation (70% saturation) and dialysed against 10 mM Tris/HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5. The protein was applied onto a phosphocellulose column (40 x 3.1 cm) also equilibrated with the same buffer. When the A_{280} of the effluent was below 0.1, the aldolase was eluted with a substrate solution containing 2.5 mM fructose-1,6-bisphosphate in the starting buffer. The flow rate was 30 ml/hr and fractions of 10 ml were collected. (|——|) represents the fractions pooled.

phosphocellulose column and polyacrylamide gel electrophoresis of the pooled fractions (Fig. 52, lane i), shows that chromatographic purification on the column resulted in an enrichment of the enzyme compared to the material recovered from the DE-52 column (lane h). The final purification step involved the use of a DEAE Sephadex A-50 column to resolve the A-C hybrid set of the tetrameric enzyme. Fig. 51 represents a typical elution profile from the anion exchange column. Upon the application of a linear NaCl gradient of 0 to 0.35 M, in 25 mM Tris/HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5, three peaks of enzymatic activity were resolved. The pooled fractions in the last peak containing the brain specific isoform of the aldolase, C_4, were essentially homogeneous by the criterion of SDS PAGE (Fig. 52, lane j). Non denaturing disc gel electrophoresis was performed and revealed that the aldolase C_4 purified using this methodology migrated with a faster mobility than a commercial preparation of muscle aldolase containing the A isoform only (not shown). Table XII summarizes the fractionation procedure of aldolase C from bovine brain. The enzyme was purified 78 fold with a specific activity of 8.9×10^3 Sigma unit / mg protein, similar to that observed for the enzyme purified by Buczylko *et al.* (1983). The enzyme was subsequently used in the *in vitro* studies to examine the effect of exogenously added S-100 protein on its activity. These studies were generally performed within three days after the enzyme had been purified.

B. Effect of S-100a Protein On the Enzymatic Activity of Aldolase C

The enzymatic activity of aldolase was determined using a reproducible, quantitative colorimetric assay. The effect of S-100a protein on the *in vitro* activity of aldolase was examined by adding a homogeneous preparation of S-100a protein that had been purified from bovine brain to the assay reagents prior to initiating the reaction by addition of the enzyme. Table XIII summarizes the results of the assays performed in the presence of S-100a protein. In these assays the lowest molar ratio of S-100a : aldolase chosen was 5:1. This was due to fact that in the presence of approximately equimolar

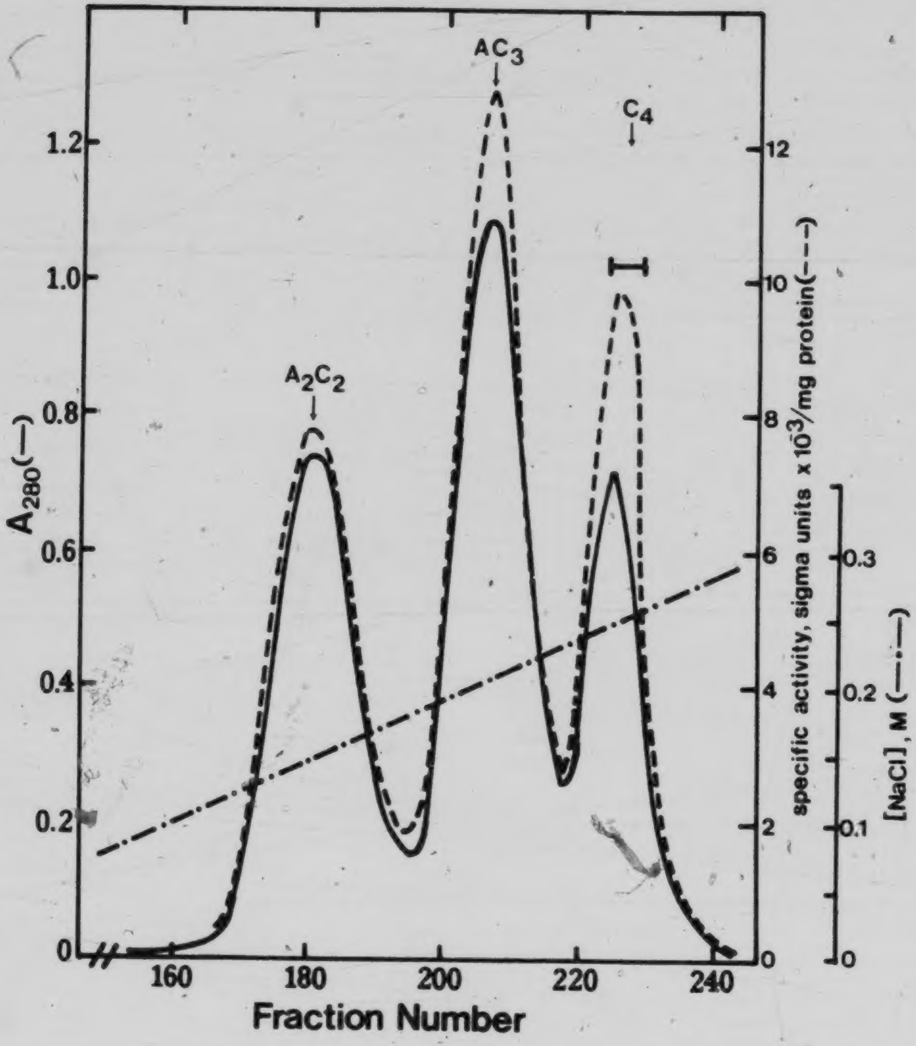

Fig. 51 DEAE Sephadex A-50 chromatography of brain aldolase. The aldolase eluted from the phosphocellulose was applied to a DEAE Sephadex A-50 column (42 x 1.5 cm) equilibrated with 25 mM Tris/HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5. The elution was carried out with a 360 ml linear NaCl gradient (0 - 0.35 M). The flow rate was 11 ml/hr and fractions of 1.5 ml were collected. (|——|) represents the fractions pooled and used in the subsequent studies to examine the effect of S-100a protein on the *in vitro* activity of the C_4 isoform of the aldolase.

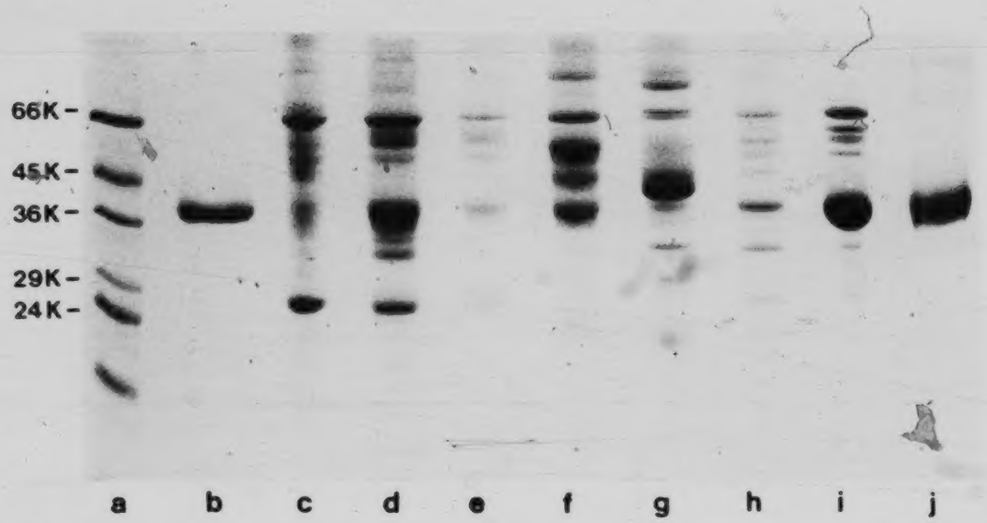

Fig. 52 SDS polyacrylamide gel electrophoresis of bovine brain aldolase obtained at various steps in the purification procedure. The lanes represent: (a) molecular weight markers; (b) muscle aldolase from a commercial source; (c) DE-52 fraction 48; (d) DE-52 fraction 66; (e) DE-52 fraction 84; (f) DE-52 fraction 100; (g) DE-52 fraction 118; (h) DE-52 cellulose column pooled fraction; (i) phosphocellulose column pooled fractions, and (j) DEAE Sephadex A-50 column pooled fractions. The concentration of acrylamide in the gel was 15%.

TABLE XII

Purification of aldolase C from bovine brain

Purification step	Total protein (mg)	Total activity (Sigma units x 10^3)	Specific activity (Sigma units x 10^3/mg protein)	Purification (fold)	Recovery (%)
25,000 g supernatant of crude extract	22,300	2565 (160)*	.115	1	100
40-60% $(NH_4)_2SO_4$ precipitate	2,831	1983	0.69	6	77
DE-52 chromatography	425	672	1.58	13.7	26
phosphocellulose chromatography	36	425	11.8	102	16
DEAE Sephadex A-50 chromatography	6.25	56	8.9	78	3

* Calculated value for aldolase C contribution. For these calculations aldolase A-C set activity ratios of 1:4:6:4:1 were assumed for A_4:A_3C:A_2C_2:AC_3:C_4, respectively.

TABLE XIII

Effect of S-100a Protein on the Enzymatic Activity* of Aldolase C

S-100a /aldolase	S-100a concentration, M	% stimulation** (EGTA)	% stimulation** (Ca^{2+})
5	8.3×10^{-9}	9.3	11.5
23	3.6×10^{-8}	11.4	18.0
52	8.3×10^{-8}	16.5	25.4
107	1.7×10^{-7}	22.5	34.3
213	3.4×10^{-7}	29.3	41.4

* Enzymatic activity was expressed in terms of A_{560}, based on the colorimetric assay as described in methodology.

** In the presence of S-100a protein, the rate of cleavage of the substrate by aldolase increased and the extent of this increase is reported as a percent stimulation of the enzymatic activity. The latter is obtained by subtracting the basal activity obtained in the absence of S-100a from that observed in the presence of S-100 protein. The results reported here represent the mean % stimulation of at least 3 measurements at each concentration of S-100a protein. The standard deviation in the extent of the stimulation of aldolase activity was ± 5.5%.

The final concentration of aldolase C was 1.61×10^{-9} M and the assay was performed in the presence of 1 mM Ca^{2+} or EGTA.

concentrations of S-100a and aldolase (1:2, 1:1 and 2:1), the change in the enzymatic activity relative to the basal activity obtained in the absence of S-100, was within the experimental error of ± 5.5%. From Table XIII, it can be seen that the *in vitro* activity of aldolase, in the presence of 5 fold excess of S-100a, increased by 9.3 and 11.5 ± 5.5% in the absence and presence of Ca^{2+}, respectively. Since the addition of either Ca^{2+} or EGTA had no effect on the enzymatic activity, the results suggested that the observed increase in the aldolase FDP cleavage activity was due to the presence of S-100a protein. At low molar ratios of S-100 : aldolase, i.e. 5:1, both the apo and Ca^{2+} bound S-100a stimulated the enzymatic activity to a similar extent, suggesting that the effect exerted by S-100 was Ca^{2+} independent. However, when the concentration of S-100a was increased to give a final molar ratio of 213:1, aldolase activity in the presence of Ca^{2+}, increased by 41.4 ± 5.5% compared to 29.3 ± 5.5% in the absence of metal ion, suggesting a small Ca^{2+} dependency of the stimulation of enzymatic activity by S-100a. Under these conditions, i.e. at the highest concentration of S-100a, the specific activity of the enzyme was 11.7 and 12.9 micromole FDP cleaved/min/mg protein, for the apo and the Ca^{2+} bound S-100a, respectively, compared to a basal specific activity of 9.1 micromole FDP cleaved/ min/mg protein. The enzymatic assays were also performed with S-100a present at a concentration similar to that found in mammalian brain. In the presence of a 780 and 1900 fold excess of the latter (i.e. final concentration of 1.3 and 3.1 μM, respectively), the aldolase activity was affected to a similar extent to that observed with the highest concentration of S-100a protein reported in Table XIII, i.e. maximal stimulation of 30 - 40% relative to the basal activity.

C. Physico-chemical Studies On The Interaction of S-100a protein with Aldolase C

Since the enzymatic assays suggested that the *in vitro* activity of aldolase C was affected by the S-100 protein, physico-chemical studies were performed to investigate the possibility that the latter might exert its effect by forming a complex with the enzyme. In the past, far UV CD has been used to demonstrate interprotein interactions such as those involving the various troponin subunits from bovine cardiac muscle (Burtnick and Kay, 1976). Hence, far UV CD spectra were recorded both in the "minus Ca^{2+}" and "plus Ca^{2+}" states of S-100a protein and aldolase C (spectra not shown). Of these spectra, only that of the S-100a protein was affected by Ca^{2+} addition, resulting in a 4.6% decrease in the $[\theta]_{222}$ value (818 ± 300 deg.cm^2.dmol^{-1}). Using these spectra, a set of calculated spect[illegible] were generated, representing each of the experimental situations subsequently tested. The calculated spectra represented linear combinations of the contribution of each protein present in the test solution. Such a spectrum should match the experimentally measured spectrum in the case where no interprotein interaction occurred. Fig. 53a represents the theoretical and experimental spectra obtained upon mixing S-100a and aldolase C in equimolar ratio in the "minus Ca^{2+}" state and Fig. 53b, the corresponding spectra for the "plus Ca^{2+}" state. The small differences in the theoretical curves obtained in the presence and absence of Ca^{2+} (-11965 and -11522 ± 300 deg.cm^2.dmol^{-1}) are due solely to $\Delta[\theta]$ induced in S-100a protein by Ca^{2+}, since the conformation of aldolase C appears not to be affected by the addition of Ca^{2+}. Irrespective of whether Ca^{2+} is present or not, the experimental spectrum is similar to the one obtained by calculation, suggesting that no interprotein interaction could be detected by CD between S-100a and aldolase. CD studies were also performed with mixtures of S-100a and aldolase C at molar ratios of 1:2 and 2:1, and the results were indicative of the absence of an interaction detectable by this technique. The possibility exists that the formation of a complex did not result in a significant change in the gross conformation of the interacting proteins, resulting in the theoretical spectrum matching the one obtained experimentally; however, this is rather unlikely.

An attempt was also made to demonstrate the possibility of interprotein interaction using affinity chromatography with S-100a coupled to Sepharose-4B. In Fig. 54, it can be

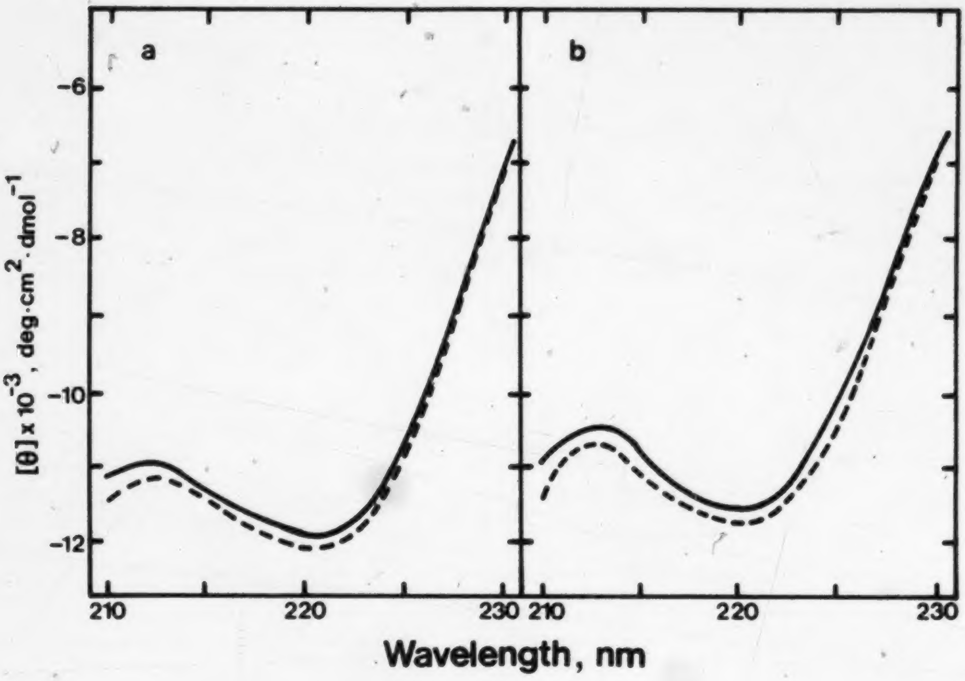

Fig. 53 Theoretical (------) and observed (——) far UV CD spectra of S-100a - aldolase C, in the presence of 1 mM EDTA (a), and in the presence of 2 mM free Ca^{2+} (b). The two proteins were dialysed separately against 20 mM Pipes, 100 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.1 and mixed in an equimolar ratio prior to the CD run. The concentrations of S-100a and aldolase C were 2.4 and 2.5 μM, respectively.

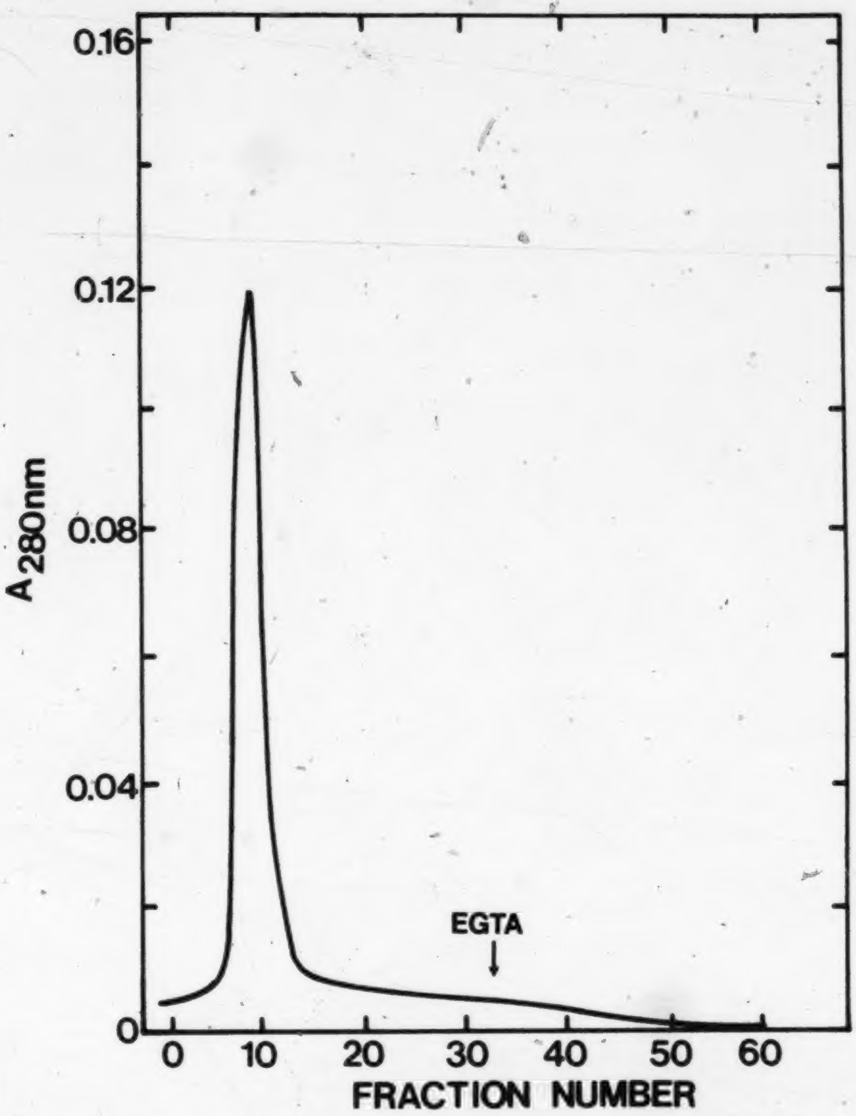

Fig. 54 Affinity chromatography of aldolase C on S-100a column. S-100a protein (16.6 mg) was coupled to CN-Br activated Sepharose-4B according to Gopalakrishna *et al.* (1985), in a buffer containing 0.1 M sodium borate,1 mM Ca^{2+}, pH 8.3. After blocking unreacted groups on the resin, the column was equilibrated with 20 mM Pipes, 50 mM NaCl, 1 mM Ca^{2+}, 5 mM 2-mercaptoethanol, pH 7.1. Aldolase C, at a concentration of 0.2 mg/ml (5 ml), after overnight dialysis against the equilibrating buffer, was applied on to the column (1 x 12.5 cm) at 8 ml/hr. At the position indicated by the arrow, the buffer was changed to 3 mM EGTA instead of 1 mM Ca^{2+}. The size of the fraction collected was 1.0 ml.

seen that upon the application of aldolase C onto a S-100a column in the presence of Ca^{2+}, essentially all the material was not retained by the column and came off in the void volume. Moreover, the subsequent application of a buffer containing 3 mM EGTA did not result in a significant amount of any additional enzyme being eluted from the column, implying that aldolase C does not bind to S-100a in the presence of Ca^{2+}. A similar result was also obtained when aldolase C was chromatographed on the S-100a column in the absence of Ca^{2+}. The ability of this column to retain S-100a binding proteins was checked by applying bovine brain homogenate to the column in the presence of Ca^{2+}. The material that bound under these conditions was eluted with EGTA, and when analysed by SDS PAGE, revealed proteins with molecular weights similar to those observed by Gopalakrishna *et al.* (1985). Hence the S-100a column was functional.

D. Discussion

The brain specific isoform of aldolase has been purified to homogeneity from bovine brain and the effect of S-100 protein on the *in vitro* activity of this enzyme investigated. Aldolase is a ubiquitous glycolytic enzyme which catalyses the reversible aldol cleavage of fructose-1,6-bisphosphate (FDP) into the triose phosphates, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. One class of the enzyme, found in animals and higher plants, requires the formation of a Schiff base with its substrate for catalysis. In mammalian tissues, the tetrameric enzyme exists in several isoforms, with aldolase A present in muscle and most other organs, whereas aldolase B is usually confined to the liver and kidney, and aldolase C is characteristic of nervous tissues. The isoenzymes have been purified to homogeneity (Penhoet *et al.*, 1969a) and shown to differ in their substrate specificity and immunological properties (Penhoet *et al.*, 1969b). In the brain, the A and C parental subunits combine to form active tetramers consisting of A_4, A_3C, A_2C_2, AC_3 and C_4 (Penhoet *et al.*, 1967; Lebherz and Rutter, 1969). The catalytic properties of the heteromeric tetramers have been shown to be indistinguishable from those of an equivalent mixture of homomers (Penhoet and Rutter, 1971).

Our study on the enzyme aldolase was initiated following a recent report that S-100 protein bound to and stimulated the *in vitro* activity of the isoenzyme specific to nervous tissue in a Ca^{2+} dependent manner (Zimmer and Van Eldik, 1986). It was anticipated that by using physico-chemical methods to characterize the molecular mechanism of S-100 interaction with aldolase, a better understanding of the functions of S-100 protein as a Ca^{2+} effector in nervous tissue could be obtained. As an initial step, the effect of S-100 protein on the enzymatic activity of aldolase was examined. Essentially, at low molar ratio of S-100a protein : aldolase (5:1), the stimulation of enzymatic activity by the apo and Ca^{2+} bound S-100a was small (9.3 and 11.5% ± 5.5%, respectively) and the effect appeared to be Ca^{2+} independent. However, in the presence of a large excess of S-100a, there was a small Ca^{2+} dependency in the stimulation of aldolase C activity and under these conditions, the maximum increase in enzymatic activity was ~ 41%. In contrast, Zimmer and Van Eldik (1986) reported that S-100 protein stimulated the *in vitro* activity of a C enriched fraction of rat brain aldolase by 2.5 fold over the basal activity in the presence of Ca^{2+} only. The reasons for the discrepancy in the data from the two laboratories are not clear but the fact that different methodologies were used in the isolation of this enzyme could be a factor. The procedures for purifying bovine brain aldolase C in our studies were based on the methodology of Buczylko *et al.* (1983), similar to the one used by Penhoet *et al.* (1969a, 1969b) in their isolation and characterization studies of aldolase C. On the other hand, Zimmer and Van Eldik (1986) isolated the enzyme from rat brain using hydroxylapatite, Affi-Gel blue and DEAE chromatography to obtain a C enriched fraction of aldolase that contained a mixture of AC_3 and C_4 isoenzymes. It is not clear whether the enzyme obtained from rat brain differs from that of cow, since only the cDNA sequence of mouse aldolase C is available (Paolella *et al.*, 1986). Moreover, even though our enzymatic assays were performed using S-100a protein ($\alpha\beta$), the results should not differ significantly from that obtained with a single S-100 isoform, since both the α- and β-subunits were reported to be equally effective in stimulating aldolase activity. As well, the different pH values at which the assays were performed (pH 7.1 and pH 8.6), does not appear to be an important factor in influencing enzymatic activity since Penhoet *et al.* (1969b) reported that the FDP cleavage activity of aldolase C was independent of pH in the region of 7 - 9.

Since the tetrameric aldolase can undergo reversible dissociation to give rise to inactive monomers (Engelhard *et al.*, 1976; Lebherz, 1972), it is possible that the diluted enzyme used in the assays (0.034 mg/ml or 0.21 μM), may undergo concentration dependent inactivation. However, a recent study investigating the stability of various aldolase isoenzymes concluded that the quaternary structure of aldolase C_4 was inherently more stable than the corresponding A_4 isoenzyme (Kent and Lebherz, 1984). Moreover, sedimentation studies performed with aldolase A did not detect any concentration dependent dissociation of the tetrameric enzyme into monomers when the enzyme concentration was varied between 0.005 mg/ml to 0.5 mg/ml (Engelhard *et al.*, 1976). Hence, the diluted aldolase C used in the assays most probably contained predominantly active tetramers.

In these studies, a 5 to 213 fold excess of S-100a protein, which corresponded to a concentration range of 8×10^{-9} M to 3×10^{-7} M, was used to examine the effect of S-100 on the enzymatic activity. Under these dilute conditions, it is possible that some of the S-100a protein may undergo dissociation and affect the extent of aldolase stimulation. However, the fact that when the S-100a concentration was increased to 10^{-6} M, i.e. to a level similar to that found in the brain, the results were not significantly different from those observed in the presence of 10^{-7} M S-100a, suggests that the maximal stimulation of enzymatic activity was limited to 30 - 40%. On the other hand, the concentration of S-100 protein used by Zimmer and Van Eldik (1986) in their assays was considerably higher (10^{-8} M to 10^{-5} M). Furthermore, the latter group reported that the S-100 protein stimulated aldolase C activity in a concentration dependent manner, with maximal stimulation (~ 2.5 fold) observed when the concentration of S-100α or S-100β was between 1 to 10 μM. However, the amount of enzyme used in the assays was not given and hence one cannot establish a molar ratio of S-100:aldolase in their study. Assuming the latter group maintained this ratio below 100:1, this would imply that a higher enzyme concentration was used and this could result in the S-100 protein exerting an effect different from that observed in our assays which were performed with diluted enzyme.

Using CD and affinity chromatography on S-100a Sepharose, the interaction of S-100 protein and aldolase has not been positively demonstrated, in contrast to the results obtained using a gel overlay technique (Zimmer and Van Eldik, 1986). A discrepancy in the results obtained using the two techniques has been reported for SM21, a smooth muscle protein which bound ^{125}I labelled tropomyosin, but failed to bind to a tropomyosin Sepharose - 4B affinity column (Lees-Miller, 1986). In the case of calmodulin, the method of iodination has been known to affect the calcium dependence of binding to ^{125}I labelled protein (Hertzberg and Van Eldik, 1987).

The concentration of aldolase C in human brain has been reported to be approximately 150 μg/g wet tissue (Willson and Thompson, 1980), similar to the S-100 protein which is present at a concentration of 200 μg/g wet tissue in the white matter of CNS (Moore, 1965). In view of our observation that the aldolase C activity was significantly stimulated only in the presence of abnormally high molar ratios of S-100a : aldolase (> 100:1), coupled with our inability to demonstrate a physical interaction between these proteins using CD and affinity chromatography, such an interaction, if it occurs, is unphysiological. Aldolase is not generally considered to be a Ca^{2+} regulated enzyme. The only reported effect of calcium on aldolase activity is a small Ca^{2+} sensitive increase in the K_m and V_{max} when the muscle enzyme binds to thin filaments (F actin / tropomyosin / troponin) (Walsh *et al.*,1977). However, no effect of calcium on brain aldolase has been observed. In general, very little is known about the physiological significance of the brain specific isoform of aldolase apart from the fact that it has the lowest maximal catalytic capacity in the glycolytic sequence in nervous tissue (Lowry and Passonneau, 1964). The question of whether the nervous tissue isoform of the enzyme is an intracellular target of S-100 protein must await further *in vivo* studies. Our data, in contrast to Zimmer and Van Eldik (1986), show only a small Ca^{2+} dependent stimulation of the *in vitro* activity of aldolase C at abnormally high molar ratios of S-100a protein : aldolase.

VIII GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The α-subunit of the bovine brain S-100a protein has been isolated from its parent molecule using anion exchange chromatography under denaturing conditions. Upon the removal of the denaturant, the α-subunit reassociates to form a homodimer with an apparent molecular weight of 21,000 under native solvent conditions. Isobe *et al.* (1983) detected an S-100a$_0$ isoform which represented 3 % of the total S-100 protein from bovine brain. The fact that the amino acid composition of the latter is identical to the α-subunit of S-100a and that it also exists as a dimer, suggests that S-100a$_0$ is probably identical to the protein isolated using the methodology described above.

In this thesis, a major effort was directed towards investigating the metal ion binding properties of the α-subunit and the results obtained from these studies have clarified some of the observed differences in connection with the effects of metal ions on the two principal isoforms, S-100a (αβ) and S-100b (ββ). The binding stoichiometry studies revealed that at pH 7.5, the α-subunit possesses one Ca^{2+} and one Zn^{2+} binding site, and the spectroscopic studies have shown that the two sites are distinct and that the metal ions induce different conformational changes upon binding to the protein.

Even though both the α- and β-subunit of the S-100 protein share a similar affinity for Ca^{2+} (apparent $K_d \sim 10^{-4}$ M, pH 7.5), and Ca^{2+} binding resulted in similar gross conformational changes, Ca^{2+} exerts different effects at the level of the microenvironment of the aromatic residues. In the case of the α-subunit, Ca^{2+} causes the single tryptophan and possibly one of the two tyrosines to move to a more polar environment, whereas the tyrosine in the β-subunit of S-100b becomes buried in the presence of Ca^{2+}. In S-100a protein, the effect of Ca^{2+} on the α-subunit of the heterodimer is modulated by the presence of the β-subunit. The subtle difference in the Ca^{2+} binding properties between the 2 subunits is also observed with the Ca^{2+} analog, Tb^{3+}, which exerted a larger effect in the microenvironment of S-100a and S-100b compared with the α homodimer. The labelling of cysteine 85α with the thiol specific fluorescent probe, Acrylodan, suggested that in the presence of Ca^{2+}, the fluorophore became more exposed to solvent. Hence, it can be concluded from these studies that the conformational change induced upon Ca^{2+} binding to the α-subunit is localized in the C-terminal region where Tyr-74, Cys-85, Phe-88 and 89 and Trp-90 are located. Moreover, the hydrophobic domain in this region (74α - 90α) probably became more exposed in the presence of Ca^{2+}, resulting in the hydrophobic fluorescent probe, TNS, binding to the α-subunit. On the other hand, the hydrophobic domains on S-100b are not significantly perturbed by Ca^{2+} (Baudier and Gerard, 1983). Furthermore, the labelling of Cys 84β of S-100b with N-(1-pyrenyl) iodoacetamide, PIA, indicated that in comparison to the apo state, the probe became less accessible in the presence of Ca^{2+}. In contrast, the same fluorophore attached to Cys 84 on the β-chain of S-100a protein, moved to a more polar environment (Mani and Kay, 1985a), suggesting that the β-subunit in the αβ heterodimer responded differently to Ca^{2+} compared to the ββ homodimer. It is possible that the subtle differences exerted by Ca^{2+} binding to the α- and β-subunit may modulate the Ca^{2+} dependent processes in the cell to different extents by influencing the manner in which the various isoforms interact with effector proteins.

In addition, the 2 subunits of S-100 also bind Zn^{2+} with different properties. Upon Zn^{2+} binding to the α-subunit, the apparent α-helical content of the protein decreases, whereas the β-subunit is not affected. Hence, the conformational change observed upon Zn^{2+} binding to S-100a protein is localized to the α-subunit. On the other hand, the spectroscopic studies indicated that the aromatic residues and the hydrophobic domains on the β-subunit of S-100a and S-100b are perturbed to a much greater extent than those observed with the α-subunit. More importantly, the 2 subunits differ in their affinities for this metal ion. The small change in the fluorescence and aromatic CD spectra observed upon Zn^{2+} binding to the α-subunit precluded a determination of an apparent K_d value using these spectroscopic techniques. However, the stoichiometry experiments indicated that the affinity of the α-subunit for this metal ion probably lies in the 10^{-4} M range, which is considerably lower than the K_d values of 10^{-6} M to 10^{-8} M reported for S-100a and S-100b (Mani and Kay, 1985a, 1985b; Baudier *et al.*, 1986). The distinct Zn^{2+} binding properties of the 2 subunits are reflected in the observation that whilst both S-100a and S-100b exerted a similar effect in inhibiting microtubule assembly, S-100a_0 ($\alpha\alpha$) was without effect (Donato *et al.*, 1985). In the family of calcium binding proteins, S-100b protein is unique in its high affinity for Zn^{2+}. Calmodulin is also known to bind Zn^{2+} (2 Zn^{2+}/ mole protein) with a lower affinity (apparent K_d, 8 x 10^{-5} M) (Baudier *et al.*, 1983b) than Ca^{2+} (K_d values ranging between 4 -18 x 10^{-6} M) (Haiech *et al.*, 1981).

The monovalent cation K^+ modulates Ca^{2+} binding to the S-100 protein. In the case of the α-subunit, the antagonistic effect of K^+ is probably the result of non-specific modulation of the Ca^{2+} binding sites by electrostatic shielding of K^+. However, K^+ appeared to be less effective in reversing the Ca^{2+} induced conformational change in the α-subunit compared to S-100a and S-100b, implying that K^+ exerts a larger effect on the β-subunit.

The α-subunit which exists as a non-covalently linked dimer under native solvent conditions is highly stable to thermal denaturation even in the absence of Ca^{2+}. The binding of Ca^{2+} to the protein has the effect of enhancing its stability to denaturation by guanidine hydrochloride. On the other hand, the chemical modification studies of the single tryptophan and possibly one of the two tyrosines suggested that the native conformation of the protein was extremely sensitive to perturbation of these residues. The fact that Trp-90 and Tyr-74 are found in a region characterized by residues with bulky side chains has led to the speculation that hydrophobic interactions may have a significant role in determining the folding of the polypeptide chain. In this respect, these studies can be augmented by more detailed investigations involving the use of hydrophobic fluorescent probes to determine if chemical modification of these residues results in altering the hydrophobic properties of the α-subunit.

A detailed characterization of the physico-chemical and metal ion binding properties of the S-100 protein is an important step towards understanding the structure and function relationship of this protein system. These studies have revealed that the two subunits differ in their Ca^{2+} and Zn^{2+} binding properties and that such distinctions may reflect some aspects of functional differences between the various isoforms of the S-100 protein. Moreover, the differential distribution of the isoforms in various human tissues, with high concentration of S-100b in the brain whereas skeletal and cardiac muscle contain predominantly S-100αα, suggests that the isoforms may exhibit tissue specific functions. The exact biological functions of the S-100 protein are not known. The presence of an EF hand Ca^{2+} binding site suggests that S-100 by analogy to calmodulin, may mediate some Ca^{2+} dependent processes in the cell by binding and modulating effector proteins. In order to act as a Ca^{2+} sensor, S-100 protein may require additional factors since its affinity for Ca^{2+}, in the presence of physiological concentrations of K^+, is approximately 2 orders of magnitude lower than that of calmodulin. In the case of S-100b, the fact that the binding of 4 Zn^{2+}/mole protein increases the affinity of the protein for Ca^{2+} (Baudier *et al.*, 1986), suggests that Zn^{2+} can be one of these factors. For the other isoforms, the interaction with target proteins may regulate its Ca^{2+} binding properties, analogous to that observed in troponin-C binding to troponin-I (Potter and Johnson, 1982). Based on the observation that Ca^{2+} binding to the α-subunit is likely to result in the exposure of the C-terminal hydrophobic domain, it is tempting to speculate that this region may serve as an interface for S-100 interaction with its effector protein. Since the S-100 protein affinity for Ca^{2+} increases upon labelling cysteine 85α and 84β with thiol specific fluorescent probes, it is possible that the interaction of S-100 with its effector protein may exert a similar effect in the local environment of the sulfhydryl group, thereby modulating the Ca^{2+} binding properties of S-100. The possibility that the S-100 protein, in particular S-100b, may participate in Zn^{2+} dependent function(s) in the brain cannot be ruled out. However, very little is known about the function of Zn^{2+}, apart from its critical role in zinc metalloproteins and enzymes and its involvement in several neurological diseases.

It is anticipated that a detailed interpretation of the spectroscopic results and conclusions presented in this thesis regarding the metal ion binding properties of the α-subunit will be aided by the availability of a three dimensional crystal structure of the S-100 protein. The latter might also reveal structural features that distinguish the S-100 protein from other members of the helix-loop-helix family of calcium binding proteins, and may provide some insights into the function of this protein. A study on the solution structure of S-100 protein using two dimensional nuclear magnetic resonance spectroscopy (NMR) will facilitate a comparison of the changes in the tertiary structure of the apo and Ca^{2+}/Zn^{2+} bound S-100 protein. By monitoring the chemical shifts of histidine resonances, ^1H NMR is particularly suited to probing the conformational change induced upon Zn^{2+} binding to the S-100 protein. Both of these approaches are now underway, in collaboration with the laboratories of Drs. M. James and B. Sykes.

In addition to proton NMR, ^{113} Cd NMR studies can be performed with the S-100 protein. A similar study on the porcine intestinal calcium binding protein revealed one resonance at -105 ppm, which is typical of an EF hand Ca^{2+} binding site. However, a second upfield shifted resonance at -155 ppm, due to the N-terminal pseudo EF hand site, was also detected (Vogel *et al.*, 1985). In the case of the S-100 protein, the presence of a cluster of basic residues in the pseudo EF hand site could result in Ca^{2+} ligands with a pK_a of 7.0 or higher, and hence it would be of interest to obtain ^{113}Cd chemical shifts for S-100 protein in order to make a comparison with EF hand sites that contain predominantly carboxylate ligands.

Based on the results obtained from the spectroscopic studies, which suggested that Ca^{2+} and Zn^{2+} bound to distinct regions on the α and β-subunit, it would be of interest to determine the distance between the 2 metal ion binding sites on the S-100. One can approach this problem by performing fluorescence measurements of the α and β-subunit with Tb^3 and Co^{2+} metal ions substituted in the Ca^{2+} and Zn^{2+} binding sites, respectively. In the case of S-100b, which binds Zn^{2+} with high affinity, the identification of the Zn^{2+} binding region(s) on the molecule could also be examined by fragmentation of the β-subunit.

With the availability of a synthetic S-100β gene (Van Eldik *et al.*, 1988) as well as cDNA clones to rat S-100α (Kuwano *et al.*, 1984) and S-100β (Dunn *et al.*, 1987), one can combine the powerful technique of site directed mutagenesis with biophysical studies to probe the structure and function relationship of the S-100 protein. In the absence of a crystal structure, it is difficult to organize a systematic approach to this problem, but the following areas may prove of benefit to further the understanding of the different metal ion binding properties between the two subunits as well as with other members of the calcium binding protein family. In the case of the α-subunit, the effect of amino acid substitutions of Asn 86 and Asn 92 with histidines might provide an answer to the question as to whether the low affinity of the αα isoform for Zn^{2+} is due to the absence of histidines corresponding to His 85 and 90 on the β-chain. Secondly, the role of the C-terminal hydrophobic domain in providing an interface for S-100 interaction with effector proteins can be examined using model compounds such as phenothiazines. In view of the unique nature of the pH dependence of Ca^{2+} binding to S-100, it would be ideal to examine the role of His 25β in this phenomenon. Finally, the N-terminal Ca^{2+} binding loop is also of considerable interest since Szebenyi and Moffat (1986) proposed that the low affinity of the S-100 protein for Ca^{2+} compared to Calbindin D_{9k} (ICaBP), is due to a lack of sequence homology in this region of the molecule. Hence, by substituting Ser 29α (or Lys 28β) with a proline residue that corresponds to Pro 20 of ICaBP and replacing Lys 30α and Lys 31α with neutrally charged residues, one can examine whether electrostatic effects as well as increased loop flexibility in the pseudo Ca^{2+} binding site are responsible for lowering the affinity of S-100 for Ca^{2+}.

In summary, despite the fact that the α- and β-subunits of S-100 protein share an extensive sequence homology, the two subunits have different metal ion binding properties. Although an attempt to identify a plausible function of the S-100 protein in the glycolytic pathway has been unsuccessful, we have obtained considerable knowledge of the physico-chemical properties of the two subunits which will be invaluable in future studies probing the structure and function relationship of the S-100 protein. It is anticipated that the bulk of future physico-chemical experiments on S-100, and mutants thereof produced by site specific mutagenesis, will focus on X-ray crystallography and two- and three-dimensional NMR which will offer the possibility of obtaining both structural and dynamic information of these proteins in solution. As well, a continuing effort will be mounted on the isolation, identification and characterization of the S-100 target proteins specific to each tissue.

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Appendix I

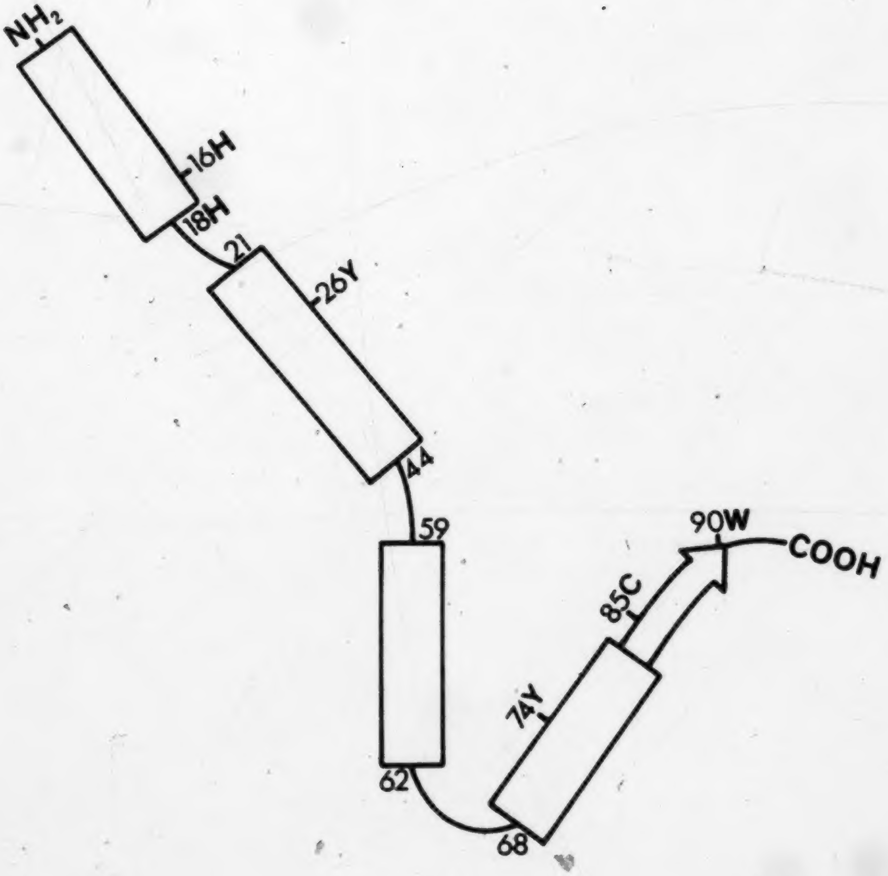
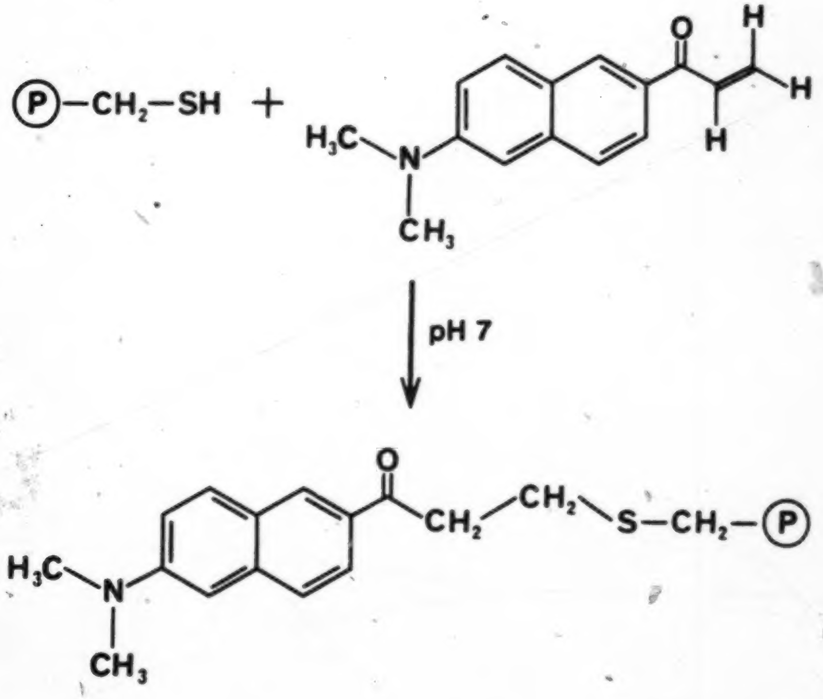


Fig. 55 Schematic diagram illustrating the secondary structure of the α-subunit predicted using the Chou and Fasman algorithm (1974) . The α-helices are represented by cylinders whereas the β-sheet and random coils are drawn as arrows and lines, respectively.

Appendix II



A mechanism proposed for the reaction of 6-Acryloyl-2-dimethylaminonaphthalene (Acrylodan) with the cysteine residue of the α-subunit of S-100a protein. The fluorescent probe which interacts specifically with sulfhydryl groups in a covalent manner, is highly sensitive to the polarity of its environment. This is attributed to the presence of a dimethylamino group at position 2 of the naphthalene ring which serves as an electron donor and a C=O group at position 6 of the ring acting as as electron acceptor.